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Swift-kinetics driven RSM optimized, biocompatible AgNPs inhibit uropathogenic biofilms via AHL interference: mechanistic insights from Ag8-SdiA in-silico docking

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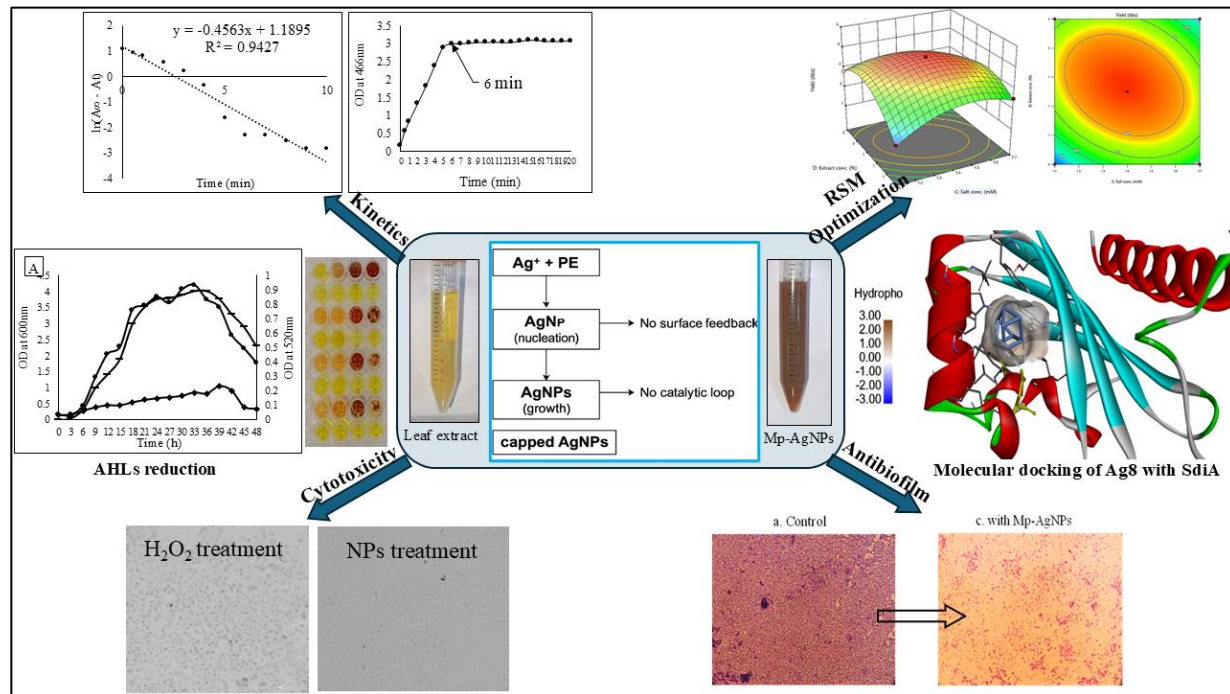
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Abstract:

Urinary Tract Infections remain a highly prevalent global health concern, with biofilm-forming, multidrug-resistant uropathogens such as Uropathogenic *Escherichia coli* (*UPEC*) and *Enterobacter hormaechei* posing significant therapeutic challenges. In this study, silver nanoparticles synthesized using *Mussaenda philippica* L. leaf extract were optimized through RSM, investigated for its rapid reaction kinetics, tested for application in quorum quenching and hence biofilm disruption against two uropathogens - *UPEC* and *E. hormaechei*. Mechanistic insights are gained by molecular docking of Ag8 nanocluster with SdiA receptor of AI-1 signals. Green synthesis was optimized using RSM, achieving high nanoparticles yield and activity under optimal conditions (pH 9, 45 °C, 1.4 mM AgNO₃, 1.125% extract). It is a pseudo-first-order behavior ($k' = 0.4463 \text{ min}^{-1}$, $R^2 = 0.94$) with a half-life of 1.55 min, indicating rapid, chemically driven Mp-AgNPs formation without lag phase. Long-term colloidal stability was observed, with unaltered SPR peaks over two months, attributed to effective phytochemical capping. Antibiofilm activity was evaluated using qualitative and quantitative assays. Mp-AgNPs inhibited biofilms by 69% in *UPEC* and 67.78% in *E. hormaechei*, and reduced EPS. Mp-AgNPs also suppressed AHLs production and cell surface hydrophobicity. Mechanistic investigation from molecular docking simulation revealed that Ag8 cluster bind within the hydrophobic cavity of SdiA regulators in both uropathogens via metal-acceptor co-ordination. This docking insights suggests that Ag8 interaction may disrupt SdiA function, providing a mechanistic basis for AgNPs mediated quorum sensing interference and biofilm inhibition. Finally, excellent biocompatibility with HaCaT cells represent Mp-AgNPs as a promising green nanotechnology-based approach to combat AMR/MDR in uropathogens.

Keywords: Biofilm, *Enterobacter hormaechei*, Molecular docking, Quorum sensing, SdiA, Silver nanoparticles, Urinary Tract Infections.

Graphical Abstract:



Highlights:

1. A high-rate constant ($k' = 0.4463 \text{ min}^{-1}$) and short half-life (1.55 min) validate one of the fastest nanoparticles biosynthesis.
2. Mp-AgNPs yield, and synthesis efficiency enhanced through RSM optimization, attributed to phytochemicals activity and not its concentration.
3. Biofilm and EPS inhibition in *UPEC* and *E. hormaechei* was mediated by AHLs suppression.
4. Ag8 cluster stably docks into hydrophobic pocket of SdiA.
5. AgNPs interacts with conserved Asp80 in *UPEC*, but target a novel site, Asp103, in *E. hormaechei*.
6. Molecular docking reveals Mp-AgNPs can target SdiA, disrupting quorum sensing.

1. Introduction

Urinary tract infections (UTIs) represent a significant and widespread global health concern, affecting an estimated 150 million individuals annually. This high incidence contributes substantially to morbidity and incurs healthcare costs exceeding \$5 billion in the United States alone [1]. While UTIs affect individuals across all age groups and genders, approximately 50% of women and 5% of men are projected to experience at least one episode in their lifetime [2]. Clinically, UTIs can manifest as urethritis, cystitis, or pyelonephritis, leading to both acute symptoms (e.g., dysuria, fever) and severe long-term complications, including permanent renal damage and scarring. Among these pathogens *UPEC* is the most common causative agent, responsible for approximately 80% of both community- and hospital-acquired UTIs. *UPEC*'s virulence is further amplified by its capacity to invade and replicate within epithelial cells, forming intracellular reservoirs that serve as critical sources of reinfection [3]. Another clinically significant pathogen is *Enterobacter hormaechei*, a member of the *Enterobacter cloacae* complex and a key component of the critical ESKAPE group. This bacterium has been increasingly associated with healthcare-related UTIs and bloodstream infections, demonstrating robust biofilm formation and extensive multidrug resistance (MDR) [4]. Compounding this issue, the widespread misuse and overuse of conventional antibiotics have propelled bacterial resistance to alarming and often untreatable levels. For instance, resistance to ampicillin and cotrimoxazole has been reported at approximately 50–57% and 23–43%, respectively, in the United States [5, 6]. A review by Kaur and Kaur [7] cited a surveillance study conducted in Pune, India, which reported resistance rates of 98% and 79% for trimethoprim/sulfamethoxazole and levofloxacin, respectively, among uropathogenic isolates. Despite continued clinical use, the therapeutic efficacy of these agents is diminishing due to the increasing prevalence of multidrug-resistant uropathogens. The World Health Organization (WHO) continues to highlight antimicrobial resistance (AMR) as one of the top global health threats, underscoring the urgent need for novel therapeutic interventions (WHO 2023).

A primary therapeutic challenge in managing UTIs stems from the ability of uropathogens to form biofilms. These are structured microbial communities embedded within an extracellular polymeric matrix, which effectively shields bacteria from both antibiotic therapies and host immune responses. This protection leads to persistent infections, frequent recurrences, and the rapid development of MDR. The enhanced resilience and adaptive capabilities of these pathogens within the host are largely attributed to quorum sensing (QS), a bacterial communication system that intricately regulates the expression of genes involved in virulence,

adhesion, and robust biofilm maturation through the production of signaling molecules. These signaling systems have been classified as Autoinducer-1 (AI-1), AI-2 and AI-3I, for intraspecies communication by gram-negative bacteria, for interspecies communication by gram-positive and gram-negative bacteria, and for interspecies, intraspecies and interkingdom (prokaryotic and eukaryotic) communications, respectively [9]. AI-1 is mainly the acyl homoserine lactones (AHLs); AI-2 are derivatives of furanosyl borate diester and derivatives of 4,5-Dihydroxy-2,3-pentanedione (DPD); and AI-3 are 3,6-dimethylpyrazin-2-one and related derivatives [10, 11] [12]. While many gram-negative bacteria employ canonical LuxI/LuxR QS systems that produce and detect specific AHL signals, which include - firstly characterized in *Vibrio fischeri*; LasI/LasR and RhlI/RhlR in *Pseudomonas aeruginosa*; CviI/CviR in *Chromobacterium violaceum*; TraI/TraR in *Agrobacterium tumefaciens*; CepI/CepR in *Burkholderia cepacia*; CerI/CerR in *Rhodobacter sphaeroides*; SinI/SinR in *Sinorhizobium meliloti* [13]; EsaI/EsaR system in *Pantoea stewartii* [14]. Certain bacterial species like *E. coli*, *Salmonella* lack LuxI or similar synthase but encode LuxR homolog, SdiA. This orphan transcription regulator enables them to ‘eavesdrop’ on AHLs produced by other species and thereby modulating biofilm formation, virulence, and antibiotic resistance through interspecies communication. Disrupting these signalling, called Quorum quenching (QQ), has been greatly explored until now as an effective strategy to inhibit the pathogens.

In light of the pressing challenges given above, silver nanoparticles (AgNPs) represent a highly promising alternative due to their potent antimicrobial and antibiofilm properties for combating the biofilms. Their nanoscale dimensions and high surface area-to-volume ratio facilitate effective interaction with microbial membranes, efficient penetration of established biofilms, and disruption of essential cellular processes [15]. Furthermore, AgNPs can function as quorum sensing inhibitors (QSIs), specifically interfering with bacterial signaling pathways to prevent biofilm formation and the expression of virulence factors in notorious pathogens such as *Proteus mirabilis*, *Klebsiella* spp., and *Pseudomonas aeruginosa* etc. [16, 17]. However, traditional chemical synthesis methods for AgNPs often involve the use of toxic substances and energy-intensive conditions, raising significant environmental and health concerns [18]. As a more sustainable and environmentally benign alternative, green synthesis using plant extracts has gained considerable attention, as evident from the literature. In this eco-friendly approach, diverse phytochemicals present in plant extracts serve as natural reducing, stabilizing, and capping agents under ambient conditions, circumventing the need for harsh chemicals or extreme energy inputs.

Green synthesis of nanoparticles using plant extracts are sustainable and has gained popularity over other methods of chemical or physical nature that often involve the use of hazardous reagents, large energy consumption, and environmentally dangerous by-products[19, 20]. Despite the increasing interest in green synthesis of AgNPs, the exploration of novel plant sources remains critical for discovering highly efficacious and biocompatible nanomaterials. *M. philippica* L., a tropical medicinal and ornamental plant, is recognized for its rich phytochemical profile and established antioxidant, antimicrobial, and anti-ulcer activities [21]. Despite its proven therapeutic potential and phytochemical richness, this plant has been significantly underutilized in nanobiotechnological applications. Our previous work demonstrated *M. philippica*'s ability to synthesize various metal nanoparticles with higher efficiency and potency of silver nanoparticles (Mp-AgNPs). The present study digs deep into this bioprocess via kinetics and RSM optimization studies and further extends the applicability of Mp-AgNPs to uropathogenic MDR bacteria - *UPEC* and *E. hormaechei*. In present study, we explored the molecular interactions between the octahedral Ag₈ nanocluster and the LuxR-type QS regulator SdiA by employing molecular docking analyses to investigate potential interference AgNPs with bacterial communication pathways central to virulence and biofilm regulation. Biomedical compatibility with HaCaT cell suggests that green-synthesized and optimized Mp-AgNPs exhibit substantial potential as novel and sustainable therapeutic agent for the treatment of persistent, biofilm-associated UTIs, offering a vital new strategy in the ongoing battle against antibiotic resistance.

2. Materials and Methodology:

Uropathogens Isolation and Characterization

Pure cultures of previously isolated uropathogens were subjected to identification and characterization through colony morphology analysis, MALDI-TOF mass spectrometry, and 16S rRNA gene sequencing. The identified uropathogens were preserved on Nutrient agar slants at 4°C for future experiments.

Kinetics of biogenesis of Mp-AgNPs

The kinetics of previously characterized biogenic Mp-AgNP synthesis were monitored in real time using UV–Visible spectrophotometry (Shimadzu, UV-1900i). Absorbance values were recorded at regular intervals immediately after the initiation of the reaction in-situ. To evaluate the reaction kinetics, the graphical method was applied. A linear plot was constructed

by plotting $\ln(A_{\infty}-A_t)$ versus time (t), where A_t is the absorbance at time t and A_{∞} is the absorbance at reaction completion [22]. The linearity of the plot was used to confirm reaction order kinetic behavior, and the slope of the resulting straight line was used to calculate the observed rate constant (k'). The half-life ($t_{1/2}$) was calculated using the expression $t_{1/2}=0.693/k'$. The corresponding absorbance ($\lambda_{\max}$ 466nm) vs time plot was also observed to assess the overall trend of nanoparticle formation during the reaction to diagnose the catalytic mechanism. The long-term colloidal stability of Mp-AgNPs was assessed by recording UV-Vis spectra at three time points: 1 h, 1 week, and 2 months after synthesis. Stability was evaluated by monitoring any changes in surface plasmon resonance (SPR) peak position, symmetry, and intensity over time.

Mp-AgNPs biosynthesis and RSM optimization

Response Surface Methodology (RSM) based Box-Behnken design (BBD) matrix, using Design-Expert (Stat-Ease, trial version 23.1.8.0) software, was employed to optimize the biosynthesis conditions for Mp-AgNPs to achieve maximize yield. The experimental design consisted of 27 runs, with each of the four selected independent variables- pH (A), temperature (B), salt concentration (C), and plant extract concentration (D)- tested at three coded levels (-1, 0, and +1). These variables were selected based on the results of preliminary optimization studies, in which they were identified as key factors influencing the synthesis of Mp-AgNPs. A distinct absorbance peak at 466 nm, noted during initial trials, served as the response parameter, acting as a reliable indicator of Mp-AgNPs yield throughout the RSM analysis. The statistical significance of the model and the contribution of individual terms were assessed using analysis of variance (ANOVA) via F -statistics, while the predictive accuracy was determined through the coefficient of determination (R^2) and adjusted R^2 values. The optimum synthesis conditions for achieving maximum Mp-AgNPs yield were identified by solving the regression equation and interpreting the response surface plots [23].

Antibiofilm assays

Microscopic Assessment of Mp-AgNPs Biofilm Inhibition

The antibiofilm potential of Mp-AgNPs against *UPEC* and *E. hormaechei* was evaluated using sterile glass slides. Sterile glass slides were placed individually in sterile Petri plates containing Nutrient Broth inoculated with the test organisms. Mp-AgNPs were added at sub-MIC concentrations to the experimental sets, while control sets contained bacterial culture without nanoparticles, and an additional set contained only PE without nanoparticles. The plates

were incubated aseptically at 37 °C for 48 h to allow biofilm development on the slides. After incubation, the slides were gently rinsed twice with sterile distilled water to remove non-adherent cells and stained with 0.4% crystal violet solution for 10 min at room temperature. Excess stains were removed by washing with distilled water, and the slides were air-dried. Biofilm formation was then observed under a light microscope at 100× magnification [24].

Qualitative Biofilm Assessment Using Congo red agar (CRA) Method

The biofilm-forming ability of *UPEC* and *E. hormaechei* was evaluated using the CRA plate assay, as described by Neihaya and Zaman [25]. Agar medium was prepared by dissolving Brain Heart Infusion (BHI) broth (37 g/L), sucrose (50 g/L), agar (10 g/L), and Congo Red stain (8 g/L) in distilled water. The Congo Red dye was prepared as a concentrated aqueous solution and autoclaved separately at 121°C for 15 min. Once autoclaved, it was combined with BHI agar and sucrose. The test organisms were incubated with Mp-AgNPs and PE treatments and then streaked onto the prepared CRA plates. The plates were incubated at 37°C for 48 h. Biofilm formation was assessed based on colony morphology and color. Strong biofilm production was indicated by black colonies with a dry crystalline consistency, whereas the non-biofilm producer strains formed pink to red colonies. The experiment was performed in triplicate and repeated three times to ensure reliability and reproducibility.

Quantitative Biofilm Inhibition Assay (BIA)

Biofilm inhibition by Mp-AgNPs was quantitatively evaluated in 96-well microtiter plate assay [26]. Each well of a sterile plate contained 180 µL of nutrient broth, 10 µL of *UPEC* or *E. hormaechei* overnight cultures, and 10 µL of Mp-AgNPs at sub-MIC concentrations. Control had 10 µL of plant extract added to the corresponding wells instead of nanoparticles, along with wells contained only the bacterial culture without any treatment. After incubation at 37 °C for 48 h, non-adherent cells were removed by washing the wells three times with PBS (0.01 M, pH 7.2). The attached biofilm was stained with 0.4% crystal violet (CV) for 10 min. Excess stain was removed by washing with distilled water, and the wells were dried at room temperature. The bound crystal violet was solubilized with 100 µL of 20% glacial acetic acid. The optical density (OD) was measured at 570 nm using a microtiter plate reader (Synergy H1, Agilent, USA).

Effect of Mp-AgNPs on EPS Production

To assess the impact of Mp-AgNPs on the production of Extracellular Polymeric Substances (EPS), the procedure described by Favre-Bonté et al. [27] was followed with

modifications. Briefly, test tubes containing 1 mL of Luria-Bertani (LB) broth were inoculated with uropathogens and incubated for 48 h in the presence of Mp-AgNPs at sub-MIC concentrations, with respective controls. After the incubation period, the contents of each test tube were washed with 0.9% NaCl. An equal volume of 5% phenol was added, followed by five volumes of concentrated H₂SO₄. The mixture was incubated in the dark for 1 h to allow for reaction. Following the incubation, the samples were centrifuged at 10,000 RPM for 20 min. The absorbance of the supernatant was measured using spectrophotometer (Shimadzu, UV-1900i) at 490 nm to quantify the EPS production.

Quorum Quenching Assessment

Quantification of Hydrophobicity Index (HI index)

The impact of Mp-AgNPs on the hydrophobicity index (HI) of the tested uropathogens was assessed by measuring hydrocarbon affinity in both treated and untreated cells, following the procedure described by Courtney et al. [28]. Briefly, 1 mL of bacterial suspension (OD₅₃₀ = 1.0) was transferred into sterile glass test tubes. Nanoparticles were added at sub-inhibitory concentrations for *UPEC* and *E. hormaechei*. An equal volume (1 mL) of analytical-grade toluene (Sigma-Aldrich) was added to each tube. The mixtures were vortexed vigorously for 2 min to facilitate contact between the bacterial cells and the hydrocarbon phase. Tubes were then left undisturbed at ambient temperature for 10 min to allow complete phase separation. Following this, the lower aqueous phase was carefully collected, and the absorbance was measured at 530 nm on spectrophotometer. Control samples without Mp-AgNPs were treated similarly. The hydrophobicity index (HI) was calculated using the following formula:

$$\text{Hydrophobicity (\%)} = [1 - (\text{OD}_{530 \text{ nm after vortexing}} / \text{OD}_{530 \text{ nm before vortexing}})] \times 100.$$

All experiments were performed in triplicate, and the mean values were used for statistical analysis.

Monitoring of AHLs levels

The quantification of AHLs in the clinical isolates was performed using a colorimetric method. The bacterial cultures were incubated at 37°C in sterile Muller-Hinton broth, with and without the addition of Mp-AgNPs. At 3 h intervals, 3 mL samples were withdrawn aseptically and centrifuged at 10,000 RPM for 15 min. The supernatant was then extracted using an equal volume of ethyl acetate. After phase separation, solvent was evaporated, and the residue was re-dissolved in a small amount of HPLC-grade acetonitrile. The solution was stored at -20°C until

further analysis. For AHLs detection, 40 μL of the extracted sample was dispensed into a 96-well microplate. Subsequently, 50 μL of a 1:1 (v/v) mixture of hydroxylamine (2 M) and NaOH (3.5 M) was added to each well. This was followed by the addition of 50 μL of a 1:1 (v/v) solution containing ferric chloride (10% w/v in 4 M HCl) and 95% ethanol. The OD was measured at 520 nm to quantify AHLs levels [29].

In-silico molecular modeling of Ag8 and SdiA

The receptor proteins selected for this study were the SdiA transcriptional regulators from *UPEC* and *E. hormaechei*. The three-dimensional structures of receptors were retrieved from the UniProt database [*UPEC*: UniProt Q8XBD0 (PDB ID- 4Y15); *E. hormaechei*: UniProt A0A6G4MLL4 (AlphaFold ID- AF-A0A6G4MLL4-F1)]. The preparation of the receptor for docking followed a well-established protocol as described in previous studies [30]. Receptor preparation was performed in AutoDock Tools 1.5.6 by adding polar hydrogens and assigning Kollman united atom charges [31]. The processed structures were saved in PDBQT format for docking.

The Ag8 nanoparticle cluster, exhibiting an octahedral nanoparticle structure with bonded interactions, was prepared in BIOVIA [32]. The structure was energy-minimized in PyRx employing the Universal Force Field (UFF). The minimized ligand was then converted into PDBQT format for docking experiments.

Docking simulations were carried out using AutoDock 1.5.6. For *E. hormaechei* SdiA, the grid box dimension was set to $126 \times 100 \times 94$ points, and for *UPEC* SdiA as $106 \times 86 \times 106$ points, with grid spacing of 0.375 Å. These dimensions were chosen to provide adequate space for ligand translation and rotation. The Lamarckian Genetic Algorithm was employed with 100 runs, a maximum of 27000 GA operation. Conformations were clustered at an RMSD tolerance of 1.0 Å, and ranked according to binding free energy (ΔG , kcal/mol) and cluster population. The lowest-energy conformations of the Ag8-SdiA (*UPEC*) and Ag8-SdiA (*E. hormaechei*) complexes were selected for further evaluation. Molecular interaction between selected docked complexes has been analysed using BIOVIA Discovery Studio Visualizer [30, 33].

Assessment of Cytotoxicity by MTT assay

The cytotoxic effects of the test sample were assessed using an MTT assay on HaCaT human keratinocytes. Cells were seeded into 96-well microtiter plates at a density of 5×10^3 cells/well and pre-incubated for 48 h to ensure attachment and growth. Following a 24 h exposure

to varying concentrations of the control and test samples, the cell culture supernatant was aspirated. MTT reagent (5 mg/mL) was then added to each well and incubated for 4 h to allow formazan crystal formation by metabolically active cells. The MTT solution was subsequently removed, and 100 μ L of acidic isopropanol (1N HCl in isopropanol) was added to dissolve the formazan crystals, with gentle agitation for 20 min. Absorbance was measured at 570 nm using a microplate reader. All experiments were performed in triplicate. Cell viability (%) was determined by using the following formula:

$$\text{Cell Viability (\%)} = \left[\frac{OD(\text{treated})}{OD(\text{control})} \right] * 100$$

Statistical Analysis

All experiments were performed in triplicate, with data analyzed using GraphPad version 10.4.1. Results are expressed as mean $\pm$ standard deviation. To determine the *p*-value between control and treated groups, an ANOVA test and an unpaired two-tailed t-test were used. A *p*-value of <0.05 was considered statistically significant.

3. Results

M. philippica L. plant was explored for the first time for the biogenic silver nanoparticles synthesis (Mp-AgNPs) and these nanoparticles were characterized extensively and assessed for their biological activity in our previous study [34]. These nanoparticles have exhibited notable antibiofilm activity against a clinically relevant uropathogenic bacterium *K. pneumoniae*, indicating their potential for biomedical applications. To deepen the understanding of the synthesis process and its mechanism; to elucidate the reaction pathway and rate of nanoparticle formation; to improve nanoparticle consistency and functional performance; to broaden the biological scope, the present study focuses on modeling the reaction kinetics; optimization of key synthesis conditions using RSM; and furthering the evaluation of antibiofilm potential against uropathogens viz. *E. coli* and *E. hormaechei*. Moreover, we present here for the first time how Ag8 interacts with SdiA, an orphan QS regulator, at molecular level to provide the fundamental understanding of the mechanism of biofilm inhibition by silver nanoparticles in-silico. Besides, cytocompatibility was assessed using a normal human cell line to establish the safety profile of Mp-AgNPs for potential therapeutic use.

Identification and Phylogenetic Analysis of Uropathogenic Isolates

Urine isolates from Indian patients suffering from non-catheter associated UTI, were named initially as SK_LB and SK_SM and subjected to identification using 16S rRNA gene sequencing. Gene sequence of SK_LB strain was subjected to a BLAST search, which revealed high similarity to *Escherichia coli* strain SCPM-O-B-7615 (GenBank Accession No. CP144926.1), and for SK_SM strain showed close similarity to *Enterobacter hormaechei* strain BW (GenBank Accession No. CP023569.1). Phylogenetic analysis further supported these findings, with SK_LB clustering within the *E. coli* clade and SK_SM aligning with *E. hormaechei* branches (Supplementary Fig. S1A and S1B). Based on the sequence homology and phylogenetic position, the strains SK_LB and SK_SM were identified and further designated as *UPEC* and *E. hormaechei*, respectively, and were tested in the present study.

Kinetics of biogenic synthesis of Mp-AgNPs

The kinetics of nanoparticle formation were evaluated by monitoring the decrease in unreacted silver ions over time. The data were fitted to the integrated pseudo-first-order rate law, expressed as: $\ln([A_{\infty} - A_t]) = -k't + \ln([A_{\infty} - A_0])$. The linear regression plot of ' $\ln(A_{\infty} - A_t)$ ' versus 'time' produced the equation $\ln(A_{\infty} - A_t) = -0.4463t + 1.1895$ with a correlation coefficient ($R^2 = 0.94$), indicating good linearity and confirming pseudo-first-order behaviour (Fig. 1a). The rate constant (k') derived from the slope was 0.4463 min^{-1} , signifying fast and efficient reduction of Ag^+ ions. The reaction half-life ($t_{1/2} = 0.693/k'$) was calculated as 1.55 min, demonstrating the ultrafast kinetics of Mp-AgNP synthesis. The in-situ 'absorbance–time' profile (Fig. 1b) displayed a distinct hyperbolic pattern with a sharp increase in absorbance immediately after extract addition and a plateau within 6 min, confirming rapid nanoparticle formation. A summary of plant-mediated AgNP syntheses and reaction parameters is presented in Table 1. Additionally, the long-term colloidal stability of Mp-AgNPs was evaluated through UV-Vis spectroscopy. The spectra recorded at 1 h, 1 week, and 2 months displayed negligible shifts in the surface plasmon resonance (SPR) peak with consistent intensity (Fig. 1c).

RSM-guided process refinement

Following the kinetic investigations, RSM was employed to statistically evaluate and optimize the synthesis of Mp-AgNPs, which is a statistical tool that allows simultaneous evaluation of multiple parameters and their interactions through a limited number of experiments. In contrast to our previous one-variable-at-a-time (OFAT) optimization approach, RSM enabled the identification of more accurate and reliable conditions for maximum nanoparticles yield. The four independent variables selected were pH (A), temperature (B), salt

concentration (C), and extract concentration (D), all previously established as essential for silver ion reduction and Mp-AgNPs stabilization. In initial trials, the best results were achieved at pH 9.0, temperature 40 °C, salt concentration 1.3 mM, and extract concentration 1.0%; however, these conditions did not consider potential interaction effects among the variables, which are possible in DoE driven RSM. BBD matrix allowed the study of the combined and interactive effects of the four parameters. Absorbance at 466 nm was used as the response variable to estimate Mp-AgNPs yield.

Model Fitting, ANOVA Analysis and Model diagnostic

The experimental design matrix, along with actual and predicted responses, is provided in Table 2. The statistical significance and adequacy of the model were evaluated through ANOVA via F-tests, and the results are summarized in Table 3. A quadratic model was recommended to be highly suitable and significant, with a model F-value of 210.92 and a p-value <0.0001. The lack-of-fit F-value was 0.21 with a p-value of 0.9121, indicating that the model fits the experimental data well without significant unexplained variations. Additionally, the model demonstrated excellent predictive capability, with a coefficient of determination R^2 of 0.996, an adjusted R^2 of 0.9912, and a predicted R^2 of 0.9822. The adequate precision ratio of 48.28, which greatly exceeds the minimum threshold of 4.0, further confirms the model's robustness and reliability.

Model Diagnostics

Model adequacy and validation of the fitted quadratic model were assessed through standard diagnostic tools. The Box–Cox transformation plot (Supplementary Fig. S2a) indicated that the optimal lambda (λ) was approximately 1.0, lying within the 95% confidence interval. This result confirmed that the original response data did not require transformation and that the assumption of constant variance was satisfied. The normal probability plot of residuals (Supplementary Fig. S2b) exhibited a near-linear pattern, supporting the assumption of normally distributed residuals. Additionally, the externally studentized residuals were mostly confined within the range of ± 2 , indicating the absence of significant outliers.

Interdependence of variables

The four selected parameters were studied at three levels each, and their individual as well as interactive effects on nanoparticle yield were analyzed through contour and 3D surface plots (Fig. 2a–f). Both type of plots displayed elliptical and saddle shapes. The interaction of extract and pH (Fig. 2a) showed that slightly alkaline conditions (pH 9 – 10) favored maximum synthesis. Similarly, the interaction between extract and temperature (Fig. 2b) indicated that moderate heating ($\sim 45^\circ\text{C}$) enhanced the reaction rate, without compromising the integrity of bioactive compounds. In the extract–salt concentration interaction (Fig. 2c), optimal AgNPs formation was observed when AgNO_3 concentration was maintained around 1.4 – 1.5 mM. The pH–temperature and pH–salt interaction plots (Fig. 2d–e) further confirmed that synthesis efficiency peaks in moderately alkaline, mid-temperature, and balanced salt environments of the tested ranges. Notably, the temperature–salt interaction (Fig. 2f) revealed that high salt combined with high temperature led to reduced absorbance.

Regression Model and Optimization Outcome

The final regression model for Mp-AgNPs yield, developed using RSM, was expressed in both coded and actual terms to describe the influence of the four variables on nanoparticle yield.

Regression Equation in Coded Factors:

$$\begin{aligned} \text{Yield} = & +3.966 + 0.211 * A + -0.003 * B + -0.014 * C + 0.175 * D + -0.1902 \\ & * AB + 0.769 * AC + 0.352 * AD + -0.784 * BC + 0.204 * BD \\ & + -0.452 * CD + -0.795 * A^2 + -0.578 * B^2 + -0.750 * C^2 \\ & + -1.036 * D^2 \end{aligned}$$

Regression Equation in Actual Factors:

$$\begin{aligned} \text{Yield} = & -35.470 + 1.857 * \text{pH} + 0.507 * \text{Temp.} + 22.318 * \text{Salt conc.} + 6.107 \\ & * \text{Extract conc.} + -0.006 * AB + 1.282 * AC + 0.281 * AD + -0.174 \\ & * BC + 0.021 * BD + -2.41 * CD + -0.198 * A^2 + -0.002 * B^2 \\ & + -8.337 * C^2 + -2.653 * D^2 \end{aligned}$$

Antibiofilm Potency Assessment

While *UPEC* is the well-established leading causative agent of uncomplicated and complicated UTIs, *E. hormaechei* is an emerging member of the *Enterobacter cloacae* complex, increasingly implicated in nosocomial and multidrug-resistant urinary infections. *E. hormaechei*, although historically underrecognized, has emerged as a notable uropathogen, particularly in catheter-associated and hospital-acquired UTIs [35]. WHO, even in 2024, has classified carbapenem-resistant *Enterobacterales*, including *Enterobacter* spp., as critical-priority pathogens due to their high resistance rates and clinical burden hereby underscoring *E. hormaechei*'s relevance as prime targets for antibiofilm intervention strategies. Moreover, there is very limited work done on phytogetic nanoparticle strategies targeting uropathogenic *E. hormaechei* biofilms in the current literature, establishing the present study as to address this pressing and previously less-unexplored critical gap. Hence, efforts are made here to evaluate the antibiofilm potential of *M. philippica* mediated silver nanoparticles against these two uropathogens. All the anti-antibiofilm and anti-QS assays were conducted at the Sub-MIC level (16 µg/mL) to ensure that the observed effects were not confounded by bacterial growth inhibition. Utilizing sub-MIC concentrations is crucial for isolating the impact of Mp-AgNPs on biofilm formation and QS without suppressing bacterial growth. Various qualitative (microscopy, congo red) and quantitative tests (biofilm inhibition, EPS content, AHLs levels, hydrophobicity index) were conducted for this purpose.

Phenotypic Biofilm Detection via CRA assay

The Congo Red Agar (CRA) assay was used to assess the biofilm-forming ability of the test organisms under different treatment conditions. Clear differences in colony morphology

were observed based on the treatment applied. In the control and the sample treated with PE alone, the colonies appeared black, indicating strong biofilm production. Conversely, colonies from the sample treated with Mp-AgNPs appeared pink, indicating inhibition of biofilm formation (Fig. 3). This differentiation is based on the principle of the CRA assay where Congo red dye binds to extracellular polysaccharides, the key components of the biofilm matrix. The absence of this color shift in the Mp-AgNP-treated sample qualitatively indicates reduced synthesis of EPS, which are essential for biofilm development.

Microscopic observation

Microscopic analysis of biofilms formed by *UPEC* and *E. hormaechei* on glass slides revealed clear structural variations among treatment sets. In both control and plant extract-treated slides, uniform and intense crystal violet staining indicated significant biofilm formation, characterized by densely adhered microbial clusters and a prominent extracellular matrix. Slides treated with Mp-AgNPs exhibited disrupted biofilm architecture, as evidenced by reduced surface coverage and weaker staining intensity. The biofilm appeared sparse and loosely organized, suggesting a compromised matrix and poor adherence of cells (Fig. 4).

Quantitative Biofilm Inhibition Assay (BIA)

The biofilm-inhibitory effect of Mp-AgNPs was evaluated against *UPEC* and *E. hormaechei* using the microtiter plate crystal violet assay. At a sub-MIC concentration (16 µg/mL), Mp-AgNPs significantly reduced biofilm formation, achieving inhibition levels of 69% in *UPEC* and 67.78% in *E. hormaechei* (Fig. 5). This substantial decrease in biofilm biomass demonstrates the effectiveness of Mp-AgNPs in interfering with biofilm development, even at sub-MIC concentrations.

Effect of Mp-AgNPs on EPS Production

Biofilm formation is primarily driven by the synthesis and secretion of EPS, which provide structural stability, mediate adhesion, and enhance resistance to environmental stressors and antibiotics. Since CRA and BIA assays suggested EPS inhibition, a quantitative assessment was performed. Mp-AgNPs, at a sub-MIC concentration (16 µg/mL), significantly suppressed EPS synthesis by 49.02% in *E. hormaechei* and 46.23% in *UPEC* compared to controls (Fig. 6).

Quantification of Hydrophobicity Index (HI index)

The microbial adhesion to hydrocarbon (MATH) assay using toluene was employed to assess the impact of Mp-AgNPs on the HI of *UPEC* and *E. hormaechei*. Untreated controls exhibited high hydrophobicity, with HI values of 62% for *UPEC* and 60% for *E. hormaechei*. Treatment with sub-inhibitory concentrations of Mp-AgNPs decreased the HI to 39% (p-value = 0.022) and 35.5% (p-value = 0.025), respectively (Fig. 7).

AHL Synthesis and Quenching Activity Profiling

Time-course analysis of AHLs levels was conducted for 48 h in both untreated and Mp-AgNP-exposed cultures. In untreated *UPEC*, AHLs levels rose steadily and peaked at 33 h with an OD_{520 nm} of 0.933 (Fig. 8A). Similarly, in *E. hormaechei*, AHLs levels remained low initially but increased sharply between 12–15 h, peaking at 33 h with an OD_{520 nm} of 0.935 (Fig. 8B). Mp-AgNP-treated cultures showed a markedly suppressed AHL profile across all time points, with *UPEC* declining to 0.232 at 39 h and *E. hormaechei* dropping to 0.299 at 33 h. Parallel OD_{600 nm} measurements showed no significant differences between treated and control cultures, confirming that QS inhibition was not due to growth inhibition or cytotoxicity.

In-silico mechanistic insights

The ag8 cluster efficiently accommodated in the SdiA protein showing binding free energy (ΔG) -2.15 kcal/mol in *E. hormaechei* SdiA and -2.05 kcal/mol in *UPEC* SdiA, indicating energetically stable binding modes across both SdiA homologues. In *E. hormaechei*, Ag8-SdiA stabilization was primarily achieved through metal-acceptor coordination, where silver atoms AG545 and AG549 coordinated with the carboxylate oxygen of ASP103:OD2, anchoring the cluster firmly within the pocket (Fig. 9a and b). Similarly, in *UPEC*, with AG545: coordinating with ASP80:OD2 (Fig. 9c and d), demonstrating the importance of specific aspartate residues in nanoparticle binding. The observed stabilization arises from a combination of metal-acceptor coordination and hydrophobic cavity accommodation. This dual mechanism enables the nanoparticle to engage chemically with specific residues while fitting precisely into a nonpolar environment, thereby reinforcing complex stability.

Assessment of Cytotoxicity by MTT assay

The assessment of the cytotoxic effects of Mp-AgNPs on human keratinocyte (HaCaT) cells revealed a favourable biocompatibility profile. Both quantitative MTT assay data and qualitative morphological observations consistently demonstrated that Mp-AgNPs exhibited only marginal dose-dependent cytotoxicity within the tested concentration range (5–80 µg/mL) after 24 h of exposure (Fig. 10). Specifically, HaCaT cell viability remained above 65% even at the highest tested concentration of 80 µg/mL, in contrast to the significant toxicity induced by the positive control, H₂O₂, which reduced viability to approximately 28%. This low cytotoxic impact was further substantiated by the lack of substantial changes in cell morphology, as HaCaT cells maintained their normal appearance and confluence following Mp-AgNP treatment, unlike the distinct damage observed in H₂O₂-treated cells.

4. Discussions

The present study explored the kinetics and optimization of biogenic synthesis of silver nanoparticles using *M. philippica* extract (Mp-AgNPs) and evaluated their antibiofilm potential against clinically relevant uropathogens, *UPEC* and *E. hormaechei*. Mechanistic insights were also gained from AHL levels monitoring and in-silico modelling. Uropathogens were genetically confirmed, showing 99.86% similarity to *E. coli* and 99.06% similarity to *E. hormaechei*, ensuring that the observed effects of Mp-AgNPs are relevant to clinically significant pathogens. Understanding the synthesis kinetics, optimizing reaction conditions, and elucidating the molecular mechanisms of biofilm inhibition are critical for advancing these nanoparticles toward biomedical applications.

As noted previously, *M. philippica* leaf extract could mediate the synthesis of silver nanoparticles at a very rapid rate, prompting an investigation into the kinetics of this biosynthetic process. To confirm and gain a deeper understanding of Mp-AgNPs biosynthesis, a diagnostic kinetic analysis was performed. The kinetic behaviour observed in the present system diverges from the sigmoidal profiles typically associated with multi-phase nanoparticle formation, such as those involving autocatalytic feedback loops [36]. The instantaneous upsurge in absorbance without a noticeable lag or acceleration phase strongly suggests that silver ion reduction proceeded through a chemically dominant route right from the onset. It is also indicative of rapid nucleation and direct reduction to saturation. This chemically driven mechanism can be attributed to the phytochemical composition of the *M. philippica* extract, which contains rich electron-donating compounds such as flavonoids, tannins, and phenolic acids (Mhavarkar and Jadhav 2025), highlighting the potency of these reducing agents. Such phytochemicals, rich in

electron-donating functional groups, enable direct and rapid reduction of Ag^+ ions, independent of nanoparticle-mediated catalytic enhancement [37]. Moreover, the prompt capping of nascent nanoparticles by stabilizing biomolecules (e.g., proteins, saponins, or polysaccharides) likely restricted surface-mediated feedback reactions by passivating the active sites on the reactive nanoparticles, thereby preventing further catalytic activity. The accelerated reaction may also be attributed to the high thermodynamic favourability of Ag^+ reduction under the given conditions, minimizing the need for intermediate nucleation or propagation phases. As summarized in Table 1, the present protocol represents one of the fastest reported biosynthesis using only 1 mM AgNO_3 and no external acceleration.

Pharmaceutical employability of Mp-AgNPs was assessed by monitoring long-term stability. The spectra recorded at 1 h, 1 week, and 2 months displayed negligible shifts in the SPR peak with consistent intensity, indicating excellent colloidal stability of the nanoparticles over time (Fig. 1c). The persistent sharpness and symmetry of the SPR peak further support sustained monodispersity and nanoscale nature of the particles, even after prolonged storage, suggesting that biomolecules present in the *M. philippica* extract effectively capped and hence stabilized Mp-AgNPs by preventing aggregation.

These mechanistic biosynthesis insights were further substantiated by the RSM optimization results. Notably, the PE concentration used in the kinetic trials (1.125%) was found to be near the optimal region (~1.25 - 1.5%) identified in RSM. Similarly, the salt concentration and pH used during kinetic measurements aligned well with the combinations found to support high yield in the RSM data, particularly around 1.4 - 1.6 mM silver nitrate and pH 9.0. Furthermore, at lower extract concentrations (e.g., 0.5%), both OD and rate were significantly reduced in the RSM matrix. Taken together, these observations reflect that the initial burst phase observed in the hyperbolic kinetics was due to efficient natural phytochemical reactivity rather than excessive reactant loading. The high model F-value (210.92) with a p-value <0.0001 and the non-significant lack-of-fit (F-value 0.21, p = 0.9121) indicate that the quadratic model reliably represents the experimental system without significant unexplained variation. The excellent predictive capability of the model, reflected by Regression- R^2 stats, support its robustness and employability for further analytical use for predicting Mp-AgNPs yield under different combinations of synthesis variables.

Among the six factor combinations studied, elevated absorbance values, indicative of higher nanoparticles yield, were observed in regions of alkaline pH (approximately pH 9) coupled with moderate salt concentrations (~1.2 - 1.4 mM), as well as at higher PE

concentrations, near 1.125%, and 45 °C temperature. The contour and 3-D plots reveal elliptical and saddle-shaped regions and surfaces, which are characteristic of significant interaction effects between variables [23]. Graphical trends confirm that Mp-AgNPs synthesis is not solely influenced by individual factors but is strongly governed by their interactive behaviour, thereby emphasizing the necessity of multifactorial optimization for enhanced nanoparticle production. Across all interactions, PE concentration consistently emerged as a critical factor. Absorbance values improved significantly as extract concentration increased up to ~1.25 – 1.5%. This confirms that reducing and capping agents in the extract directly influence nanoparticle formation and validates the extract level (1.125%) chosen for kinetic studies. It yielded high but not saturated absorbance, indicating proximity to the optimal range. It also substantiates that the activity of phytochemicals is influenced by the pH, temp. and salt concentration conditions. Regression and actual-factor equations too helped to evaluate the relative influence of each variable. Among the linear terms, pH (A) and extract concentration (D) had a significant positive effect on yield, while interaction terms like AC and AD also showed a synergistic impact. All four quadratic terms were negative, suggesting a curvature effect where both very low and very high levels reduced yield. The actual-factor equation enables real-world prediction of yield based on experimental input levels. As evident, pH and PE concentration had the highest coefficients, aligning with the coded model.

Overall, the RSM results support the conclusion from the kinetics study that the reaction is chemically driven and highly dependent on extract and salt concentration balance, rather than driven by autocatalytic surface interactions. This also explains the hyperbolic reaction profile where saturation is reached quickly once optimal conditions are met, confirming that plant extract-rich systems with well-tuned parameters promote fast, stable nanoparticle formation.

Conclusively, the RSM outcomes reinforce that the synthesis followed a pseudo-first-order, non-autocatalytic pathway driven by phytochemical reduction, effectively linking reaction mechanism and optimization.

Biomedical application of Mp-AgNPs was assessed by carrying out various assays to trace the antibiofilm potential against two uropathogens – *UPEC* and *E. hormaechei* and to analyse the mechanism of antibiofilm activity of optimized Mp-AgNPs. Nanoparticle treated samples displayed significant difference in the morphology of bacterial colonies seen in CRA assay and significantly different biofilm architecture noticed at microscopic level with crystal violet staining. Similarly, quantitative assays parallely reflected on these qualitative results, underscoring antibiofilm effects on statistical grounds. The observed reductions in biofilm were

marked by the p-value of 0.02 and 0.028 along with statistically significant reductions in EPS matrix with p-value of 0.032 and 0.025 for *UPEC* and *E. hormaechei* respectively for both parameters. This observed reduction in EPS production highlights a key mechanism by which Mp-AgNPs inhibited the biofilm formation, since EPS are the key structural architecture of the biofilms. Same is substantiated with the structural disruption observed in microscopic images. This observed attenuated biofilm structure in Mp-AgNP-treated samples was thought to have resulted from interference with initial adhesion processes or disruption of biofilm maturation pathways. Such effects are often attributed to the generation of reactive oxygen species (ROS), alteration of surface charge, and nanoparticle interaction with membrane-bound proteins critical to sessile growth [38]. Silver nanoparticles are known to interact with the EPS matrix, facilitating penetration into the biofilm and destabilizing its structure [39]. Once inside the biofilm, AgNPs may interfere with bacterial metabolism by disrupting electron transport processes and respiratory enzymes, leading to reduced ATP production, oxidative stress, and cell death [40]. In another study, [41] demonstrated that silver nanoparticles disrupt biofilm architecture in *P. aeruginosa* and *Staphylococcus epidermidis* by impairing cell surface interactions and inhibiting structural stability. So, AgNPs not only disrupt biofilm architecture but also attenuate virulence at sub-inhibitory concentrations, highlighting their potential as anti-virulence agents for treating biofilm-associated, multidrug-resistant uropathogens [42].

Plant-mediated synthesis may further enhance the efficacy of AgNPs due to the presence of bioactive phytochemicals, which could synergistically inhibit virulence factors [43].

Cell surface hydrophobicity (CSH) is a critical aspect for the pathogens, including uropathogens, as it governs the first step in bacterial infection which is cell surface adhesion. It has been observed that ESBL bacteria possesses higher CSH activity. Higher hydrophobicity possessing bacteria are found to adhere readily to both biotic and abiotic surfaces, supporting the establishment of dense biofilms—a characteristic strongly associated with enhanced resistance to antibiotics and immune clearance [44]. Hence, it was imperative to test this property of isolated urine cultures. Here, the significant reduction in bacterial surface hydrophobicity indicates that Mp-AgNPs disrupt early-stage biofilm formation by tempering the CSH of these bacteria and supported the earlier prediction of biofilm formation inhibition mechanism. Hydrophobic interactions are largely mediated by surface-associated molecules such as lipopolysaccharides, outer membrane proteins, and cell wall-associated fatty acids with host cell surfaces. By decreasing such interactions, Mp-AgNPs might have weakened bacterial attachment, thereby impairing biofilm initiation. Similar effects have been reported with zinc

oxide nanoparticles, which reduced both hydrophobicity and EPS production in *Pseudomonas aeruginosa* [45].

EPS (and hence the biofilm) synthesis is tightly regulated by quorum sensing and environmental cues, making it a critical target for antibiofilm strategies ([46]. Bacteria use various signalling molecules for the interspecies communication and intraspecies indications. AHLs represents the intraspecies AI-1 signalling system of gram-negative bacteria. Monitoring the levels of AHLs can clearly reveal the QS based biofilm formation activity in the cultures. The substantial reduction in AHL levels caused by Mp-AgNPs demonstrates effective QS pathways disruption in both *UPEC* and *E. hormaechei*. By targeting bacterial communication rather than growth, Mp-AgNPs offer a non-bactericidal strategy to attenuate pathogenicity and biofilm formation, minimizing selective pressure for resistance. While AHLs production in *UPEC* has been previously reported [47], present study may represent the first qualitative detection of AHLs in uropathogenic *E. hormaechei*, a member of the *Enterobacter cloacae* complex, which utilizes short-chain AHLs and AI-2 signalling for intra- and inter-species communication. These signalling mechanisms regulate coordinated behaviours, including virulence expression and biofilm development [48]; [49].

Nanoparticles can overcome complexity and challenges posed by biofilm because of their size, shape, surface charges, etc. It allows them to penetrate readily, which is further facilitated by electrostatic and hydrophobic interactions with bacterial membranes. Recent studies have shown that AgNPs exert their effects at genetic levels, modulating *UPEC* virulence genes involved in adhesion and biofilm formation viz. - FimH, Sfa, and IutA [50]. Another study [51] showed that AgNPs downregulate key biofilm-related genes, including fimA, papG, and luxS, in *UPEC*, thereby reducing adhesion and biofilm maturation. Hemag [52] reported that AgNPs impair biofilm formation by altering metabolic processes and interfering with regulatory gene networks.

In line with earlier studies, our experimental investigations here shown that the antibiofilm activity exhibited by Mp-AgNPs likely involves multiple mechanisms including the penetration and interactions at molecular level. To understand the molecular mechanism of Mp-AgNPs, we performed the molecular docking analysis of SdiA from *UPEC* and *E. hormaechei* with Ag₈ nanoclusters. Docking analysis revealed that the silver nanoparticles form stable complexes with the SdiA, a transcriptional regulator of *E. hormaechei* and *UPEC*. In both cases, the nanoparticle was accommodated within the hydrophobic cavity of the protein, indicating

strong structural and energetic complementarity between the cluster and the receptor binding site.

SdiA is a canonical LuxR-type receptor and functions as an orphan QS regulator in Enterobacteriaceae, “eavesdropping” on AHL signals produced by other species to regulate community behaviour [53]. Its C-terminal helix-turn-helix motif binds DNA, and the N-terminal domain binds autoinducers [54]. Its role in biofilm regulation is context-dependent: it can repress or activate biofilm-related genes like *csgD*, *csgA*, *fimA*, and flagellar genes depending on environmental AHL signalling [10]; [55]; ; [56]. While biofilm formation genes and SdiA are well established in *E. coli*, presence of *fimA*, *csgA*, *csgD*, and *sdiA* in *E. hormaechei* has only recently been confirmed [35]. Interactions of Ag8 with SdiA residue ASP80 in *UPEC* and ASP103 in *E. hormaechei* evident in the present investigation suggest that AgNPs may mimic AHL signals, competitively engaging the ligand-binding domain, and negatively modulate QS-mediated biofilm formation. Previous studies on SdiA have demonstrated that W67, D80, Y71, R60, and S134 residues are conserved for AHL-binding pockets in *E. coli*, *Salmonella*, and *Klebsiella*. Molecular docking studies in *Salmonella typhimurium* and *EHEC* also highlighted conserved residues involved in hydrogen bonding and hydrophobic interactions, supporting the functional significance of these residues in QS modulation. One of the earliest studies with SdiA showed that the C8-HSL molecule binds in a deep pocket in the hydrophobic core and acts as a folding switch in *E. coli* SdiA [54]; [57]; [58]; [59]; [60]. Present investigation supports the earlier reported role of conserved Asp80 for ligand binding to SdiA in *UPEC* while it highlights and suggests the ligand binding role of Asp103 in *E. hormaechei*. This residue may represent a novel binding site, expanding the versatility of LuxR-type regulators.

Overall, these findings provide mechanistic insight into how silver nanoclusters may interact with SdiA, suggesting their potential as modulators of bacterial communication, QS, and biofilm-associated virulence. The dual mechanism of metal-acceptor coordination and hydrophobic cavity accommodation may hinder conformational flexibility of SdiA, offering a strategy for molecular targeting in biofilm-associated infections.

Cell viability assays are paramount in toxicological assessments, providing critical insights into cellular responses, including survival, death, and metabolic integrity. Nanoparticles, including silver nanoparticles (AgNPs), are extensively studied for their cytotoxicity, yet reported effects on mammalian cells vary significantly across studies, often depending on synthesis methods, size, surface chemistry, and cell type [61]. For instance, while some studies report cytotoxicity of AgNPs on various cell lines, our findings for Mp-AgNPs on HaCaT cells

align with a growing body of evidence suggesting that certain nanoparticle formulations can exhibit low toxicity profiles. This intrinsic low cellular toxicity is a critical characteristic for the development of any material intended for biological interfaces, underscoring the biocompatibility of the synthesized Mp-AgNPs.

5. Conclusions

This study presents a comprehensive approach to the green synthesis of silver metal nanoparticles (Mp-AgNPs) using *M. philippica* leaf extract. Kinetic analysis revealed a pseudo-first-order, non-autocatalytic reduction process with a rapid half-life, offering mechanistic insight into the swift nucleation and growth dynamics of Mp-AgNPs. RSM-based process optimization further enhanced nanoparticle yield, monodispersity, and colloidal stability - laying a strong foundation for reproducible and scalable nanoparticles production.

This study underpins the clinical significance of *UPEC* and *E. hormaechei* as key targets in tackling MDR and biofilm-associated complications. Antibiofilm activity exhibited by Mp-AgNPs was due to multiple mechanisms viz. – disruption of initial adhesion; QQ mediated interruption of AHLs synthesis; reduction in EPS; and probable SdiA interactions. These non-bactericidal QQ mode of action, makes Mp-AgNPs advantageous for avoiding resistance development and promoting safe in vivo applicability.

In silico docking study identified that Ag8 nanoclusters bind in hydrophobic pocket of SdiA, specifically to the conserved Asp80 residue in *E. coli* but engage an apparently novel binding site at Asp103 within *E. hormaechei*. It opens new avenues to experimental validation and to the generation of species-specific antibiofilm agents that inhibit LuxR-type quorum sensing regulators. Such binding is indicative that AgNPs could resemble natural AHL ligands and therefore may compete by binding within the ligand-binding domain of SdiA and thus alter QS pathways responsible for regulation of the biofilms.

Finally, cytotoxicity studies on human keratinocytes (HaCaT cells) confirmed excellent biocompatibility at therapeutic doses, marking their biomedical potential.

To deepen the mechanistic understanding of these effects, future studies should focus on unravelling the molecular pathways modulated by AgNPs at molecular; particularly those involved in QS and biofilm regulation. This would not only elucidate their exact mode of action but also facilitate rational design of next-generation nanotherapeutics.

Authors declare that they have NOT used any Generative AI & AI tools to create or alter images.

CRedit authorship contribution statement

S.R.M.: Experimental and computational work, Writing original draft, Methodology, Investigation, Formal analysis, Data curation. S.H.B.: Molecules preparation, In-Silico studies Supervision, Manuscript editing. K.J.: Computational methodology and analysis Manuscript writing, Methodology. S.B.J.: Manuscript – Writing, review & editing, Data analysis, Validation, Supervision, Project administration, Conceptualization.

Declaration about generative AI & AI tools

Authors declare that they have NOT used any Generative AI & AI tools to create or alter images of this manuscript.

Ethical declaration

Uropathogenic strains used in this study were obtained as anonymized clinical isolates from urine samples processed in a diagnostic laboratory. No identifiable patient information was used and no direct human participation was occurred. The Institutional ethical approval is not applicable in this case.

Funding Declaration

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Declaration

All the data generated during this study is are included in this article and its supplementary information files. Further queries can be addressed to the Corresponding author.

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Table 1. Summary of plant-mediated AgNP synthesis with varied reaction conditions and times using 1 mM AgNO₃.

Sr. No.	Plant Extract Used	PE: Salt Ratio (v/v)	Reaction Temp.	Observed λ_{max} (nm)	Reported PE Concentration	Normalized Concentration	Synthesis Time	Reference
1	<i>M. philippica</i>	1:10	45°C	466 nm	1.125 g in 100 mL	1.125%	6 min	Present Study
2	<i>O. vulgare</i>	1:9	75–85°C	445 nm	10 g in 100 mL	10%	15 min	[62]
3	<i>C. angustifolia</i>	1:9	RT	420 nm	10 g in 100 mL	10%	10 min	[63]
4	<i>C. arabica</i>	1:4	100°C	450 nm	10 g in 250 mL	4%	10 min	[64]
5	<i>A. dentata</i>	1:9	RT	430 nm	10 mL in 100 mL	10%	10 min	[65]
6	<i>V. agnus castus</i>	1:9	Sunlight	450 nm	10 g in 100 mL	10%	30 min	[66]
7	<i>D. lablab</i>	1:5	80°C	426 nm	1g in 100 mL	1%	1 h	[67]
8	<i>C. guianensis</i>	1:9	60°C	432-444 nm	5 ml in 50 mL	10%	~1 h	[68]
9	<i>A. indica</i>	1:1	70°C	420-440 nm	1 g in 10 mL	10%	3 h	[69]
10	<i>S. trilobatum</i>	1:9	70°C	440 nm	10 g in 100 mL	10%	16 h	[70]
11	<i>C. longa</i>	1: 2	25°C	415 nm	0.1g in 20 mL	0.5%	24 h	[71]
12	<i>A. cyanophylla</i>	1:9	35°C	460 nm	5g in 100 mL	5%	48 h	[72]

RT = Room Temperature

Table 2. Box–Behnken design matrix, along with variables, actual and predicted response of Mp-AgNPs yield values.

Std order	A: pH (Units)	B: Temperature (°C)	C: Salt concentration (mM)	Extract concentration (%)	Actual Value	Predicted Value	Externally Studentized Residuals
1	11	45	1.7	1.125	3.32	3.39	-1.357
2	7	30	1.4	1.125	2.25	2.2	1.081
3	9	60	1.4	0.5	1.95	1.97	-0.336
4	9	30	1.7	1.125	3.45	3.41	0.733
5	9	60	1.1	1.125	3.39	3.43	-0.828
6	11	60	1.4	1.125	2.61	2.61	0.009
7	9	45	1.7	1.75	1.88	1.89	-0.15
8	11	45	1.4	0.5	1.76	1.82	-1.159
9	9	60	1.7	1.125	1.9	1.83	1.322
10	11	45	1.1	1.125	1.93	1.88	1.089
11	7	45	1.1	1.125	3.01	2.99	0.308
12	9	30	1.1	1.125	1.8	1.87	-1.433
13	7	45	1.4	1.75	1.8	1.75	1.056
14	9	45	1.4	1.125	4.03	3.97	0.979
15	9	60	1.4	1.75	2.69	2.73	-0.719
16	9	45	1.1	0.5	1.63	1.57	1.272
17	9	45	1.7	0.5	2.52	2.44	1.608
18	7	45	1.7	1.125	1.32	1.43	-2.477
19	7	45	1.4	0.5	2.05	2.1	-1.002
20	9	30	1.4	0.5	2.37	2.39	-0.248
21	9	45	1.1	1.75	2.8	2.82	-0.413
22	9	30	1.4	1.75	2.29	2.33	-0.627
23	11	30	1.4	1.125	3.02	3	0.438
24	11	45	1.4	1.75	2.92	2.87	0.904
25	9	45	1.4	1.125	3.87	3.97	-1.589
26	9	45	1.4	1.125	4	3.97	0.5
27	7	60	1.4	1.125	2.6	2.57	0.613

Table 3. Analysis of variance (ANOVA) for the recommended quadratic model.

Source	Sum of Squares	df	Mean Square	<i>F</i> -value	<i>p</i> -value	
Model	14.71	14	1.05	210.92	< 0.0001	significant
A-pH	0.4852	1	0.4852	97.41	< 0.0001	
B-Temp.	0	1	0	0.007	0.9346	
C-Salt conc.	0.0142	1	0.0142	2.85	0.117	
D-Extract conc.	0.4616	1	0.4616	92.66	< 0.0001	
AB	0.1227	1	0.1227	24.63	0.0003	
AC	2.39	1	2.39	479.83	< 0.0001	
AD	0.6679	1	0.6679	134.08	< 0.0001	
BC	2.32	1	2.32	465.35	< 0.0001	
BD	0.2079	1	0.2079	41.74	< 0.0001	
CD	0.7482	1	0.7482	150.21	< 0.0001	
A ²	3.15	1	3.15	631.52	< 0.0001	
B ²	1.76	1	1.76	353.61	< 0.0001	
C ²	3.11	1	3.11	623.53	< 0.0001	
D ²	5.52	1	5.52	1107.81	< 0.0001	
Residual	0.0598	12	0.005			
Lack of Fit	0.0368	10	0.0037	0.3194	0.9121	not significant
Pure Error	0.023	2	0.0115			
Cor Total	14.77	26				

df = Degrees of freedom

Figure Captions

Fig. 1. UV-visible spectra of Mp-AgNPs showing - a) plot of $\ln(A_{\infty} - A_t)$ Vs time depicting the kinetic investigation of Mp-AgNPs synthesis, b) plot of $\lambda_{\max}$ Vs time, indicates progress of synthesis, and c) long-term colloidal stability of the biosynthesized Mp-AgNPs.

Fig. 2. RSM 3D-surface and 2D contour plots showing the interactive effect of each variable on silver nanoparticle synthesis and yield: (a) Extract conc.- Salt conc., (b) Extract conc.- pH, (c) Temperature–pH, (d) Salt conc.- pH, (e) Extract conc.- Temperature, (f) Salt conc.- Temperature.

Fig. 3. CRA assay illustrating the effect of Mp-AgNPs on EPS production and biofilm formation. (A) *UPEC* and (B) *E. hormaechei*: (a) Control showing black, dry crystalline colonies (b) treatment with plant extract showing similar black crystalline colonies (c) treatment with Mp-AgNPs resulting in pink, mucoid colonies.

Fig. 4. Effect of Mp-AgNPs on biofilm formation observed under light microscopy. (A) *UPEC* and (B) *E. hormaechei*: (a) Control (b) biofilm treated with plant extract (PE) and (c) biofilm treated with Mp-AgNPs.

Fig. 5. Effect of Mp-AgNPs on biofilm formation in *UPEC* and *E. hormaechei*, as assessed by quantitative BIA. Data are expressed as mean $\pm$ standard deviation (SD). (*) indicates statistically significant at ($p \leq 0.05$).

Fig. 6. Quantification of EPS production in *UPEC* and *E. hormaechei* following treatment with plant extract (PE) and Mp-AgNPs. Data are expressed as mean $\pm$ standard deviation (SD). (*) indicates statistical significance at ($p \leq 0.05$).

Fig. 7. Effect of Mp-AgNPs on cell surface hydrophobicity of two uropathogens. Data are expressed as mean $\pm$ SD. * indicates significant at $p \leq 0.05$.

Fig. 8. AHLs activity in the presence and absence of Mp-AgNPs in (A) *UPEC* and (B) *E. hormaechei*. AHLs production was measured every 3 h for 48 h using a colorimetric assay (OD_{520}).

Fig. 9. Molecular docking interaction of Ag8 with SdiA protein of *E. hormaechei* and *UPEC*. visualization of the hydrophobic pocket with a hydrophobicity scale (a & c), and Docking poses showing binding orientation (b & d), respectively.

Fig. 10. Assessment of the cytotoxicity of Mp-AgNPs in MTT assay using HaCaT cells. (A) Dose-dependent effect of Mp-AgNPs on cell viability (%) assessed by MTT assay. (B) Light microscopic images of HaCaT cells after 24 h exposure to: (a) untreated control, (b) positive control (250 μ M of H₂O₂), (c) 5 μ g/mL Mp-AgNPs, (d) 10 μ g/mL, (e) 20 μ g/mL, (f) 40 μ g/mL, (g) 80 μ g/mL. Data are presented as mean $\pm$ SD* indicates significant at $p \leq 0.05$.

Fig. 1(a-c)

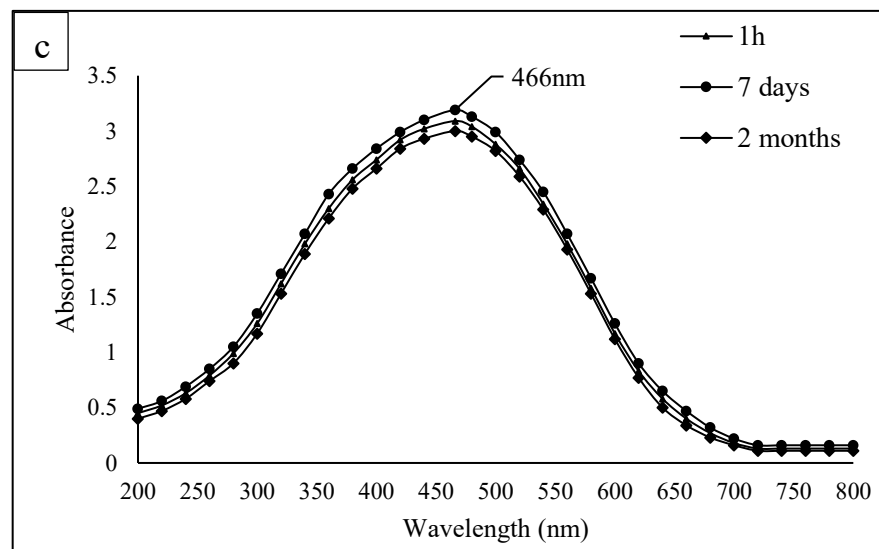
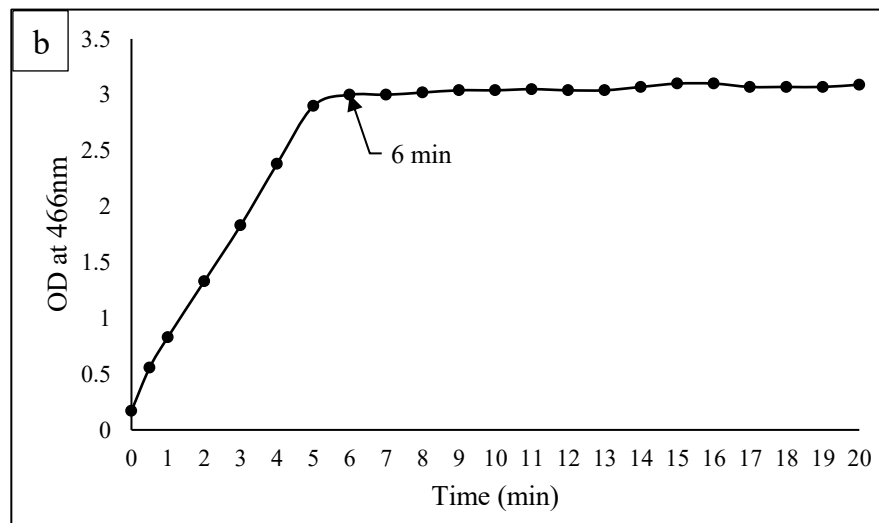
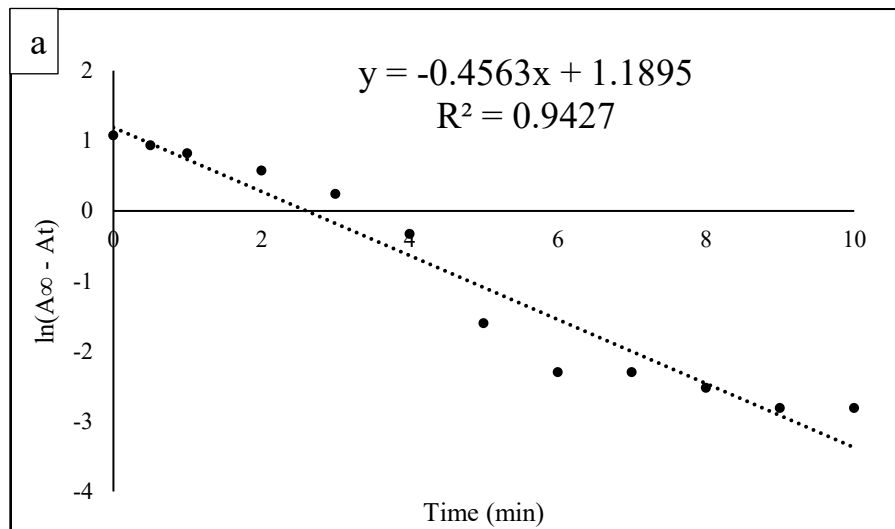


Fig. 2(a-c)

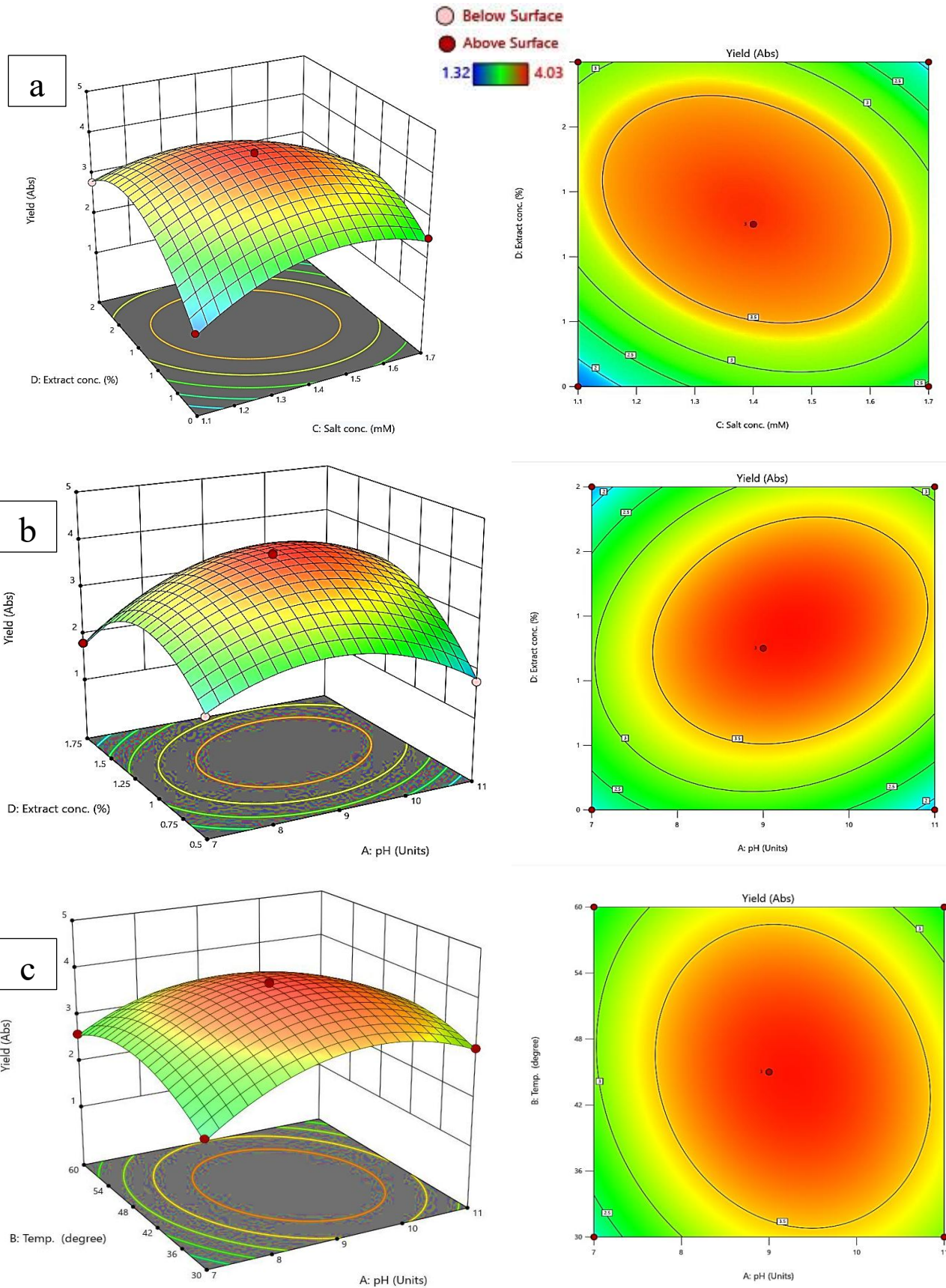


Fig. 2(d-f)

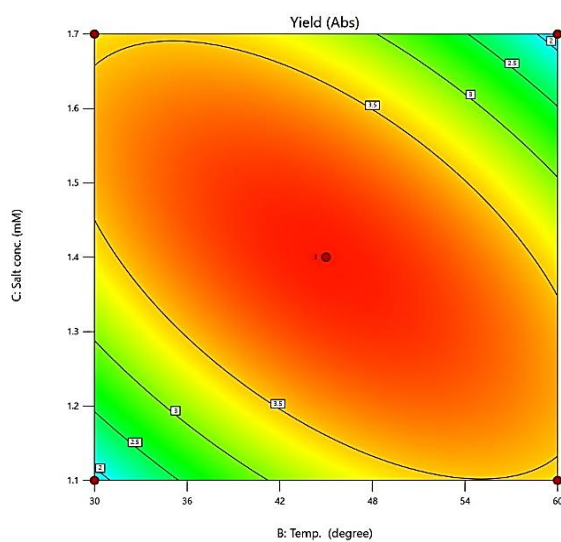
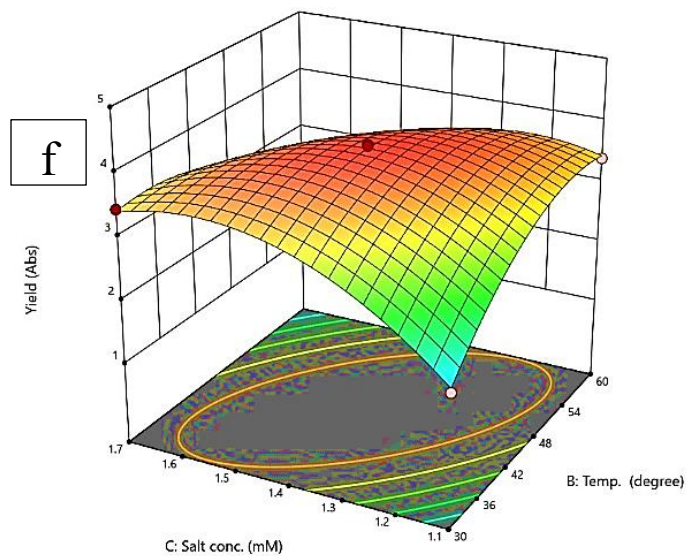
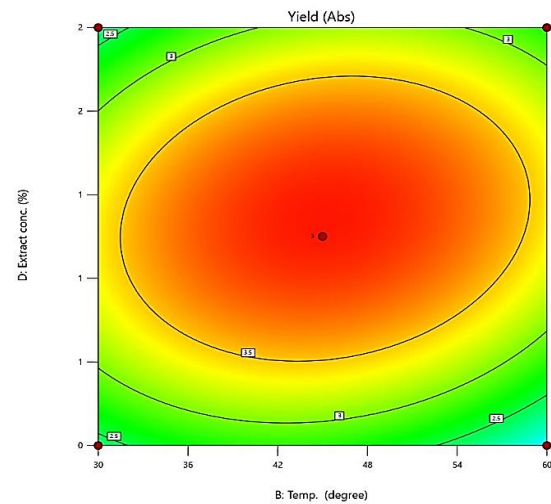
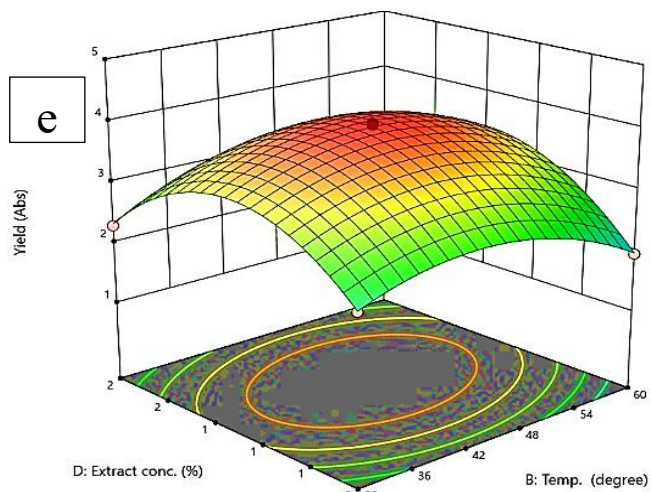
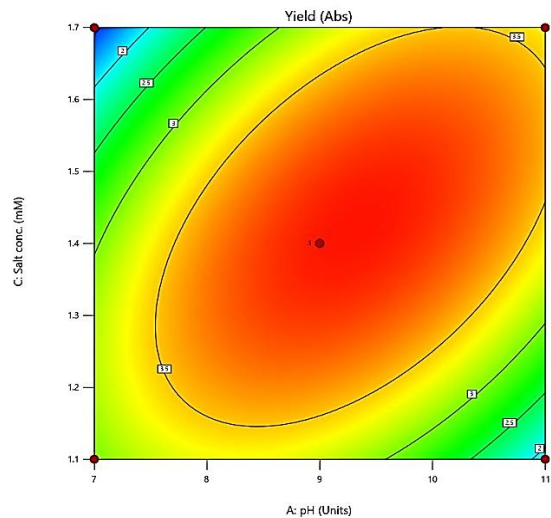
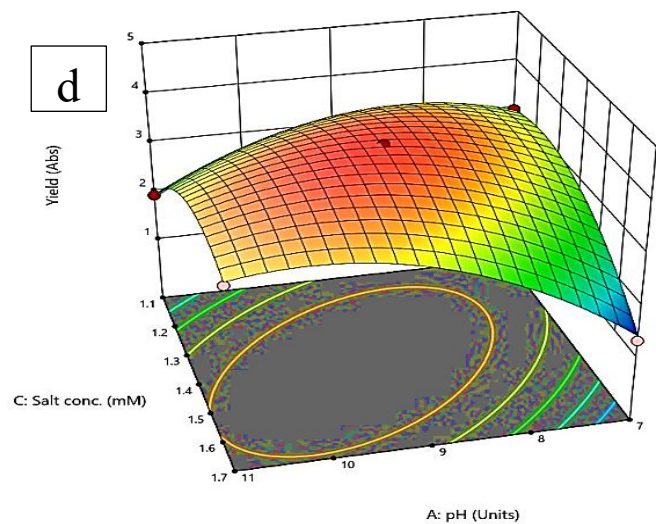


Fig. 3

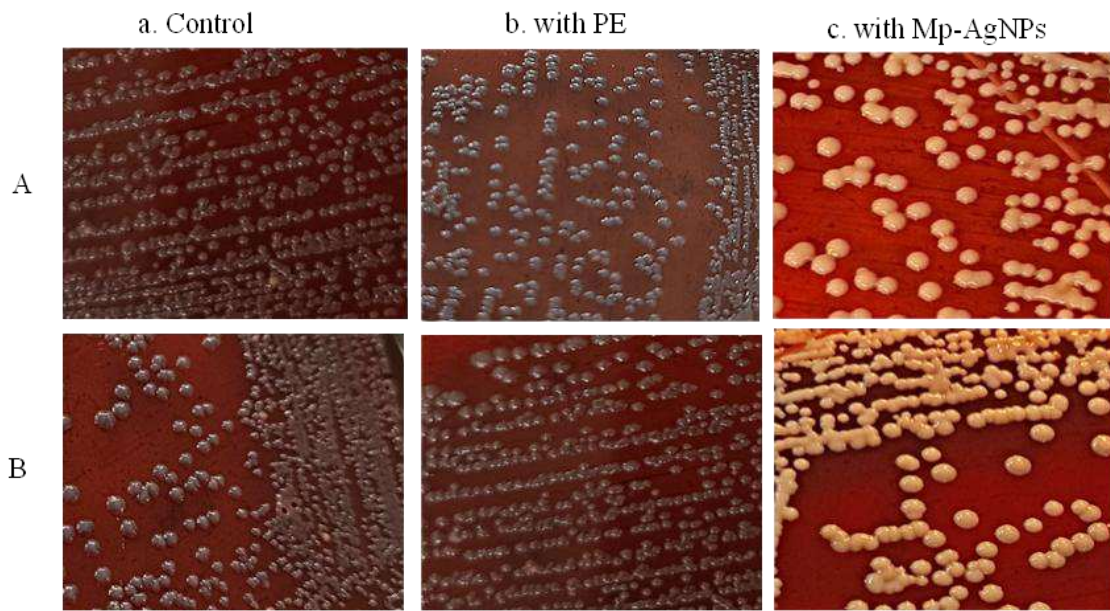


Fig. 4

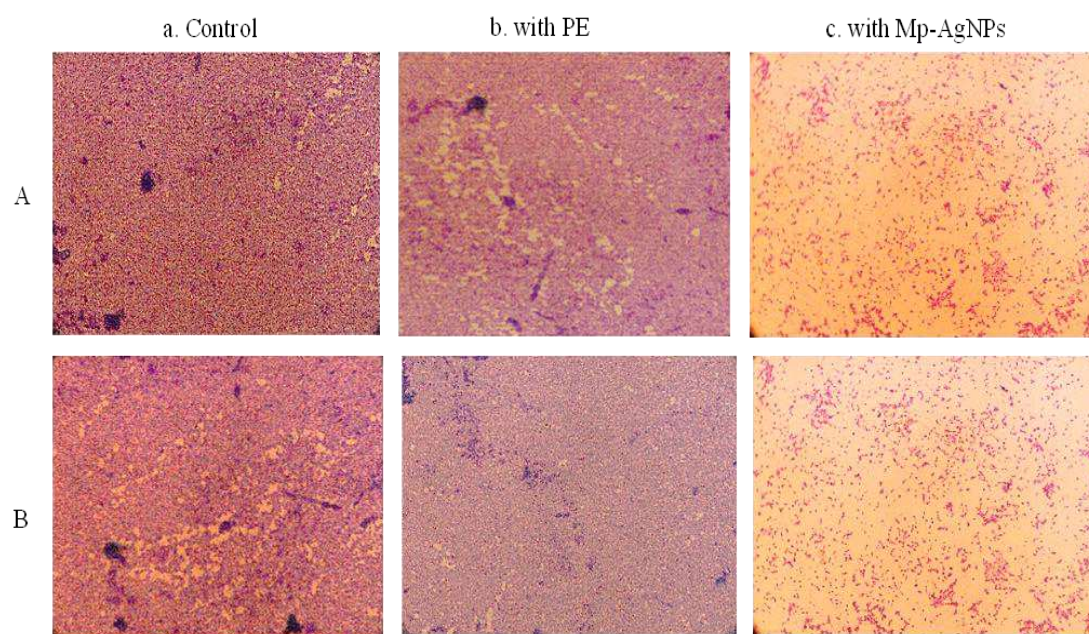


Fig. 5

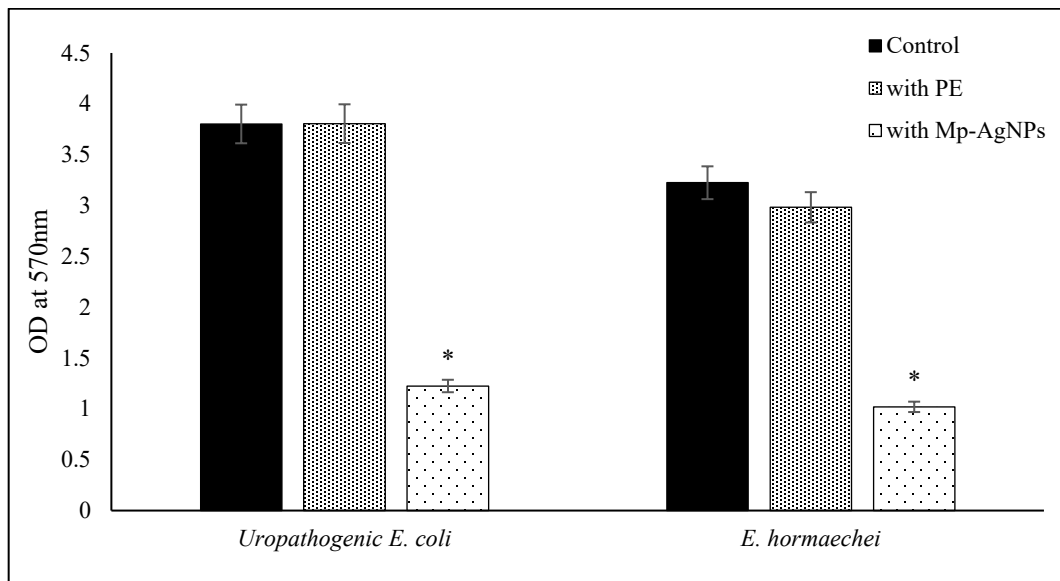


Fig. 6

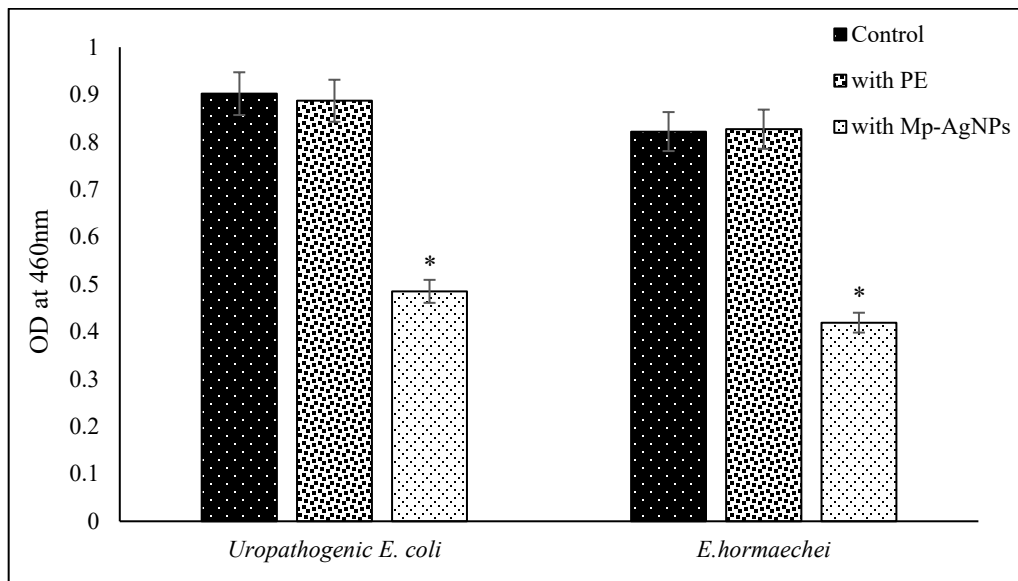


Fig. 7

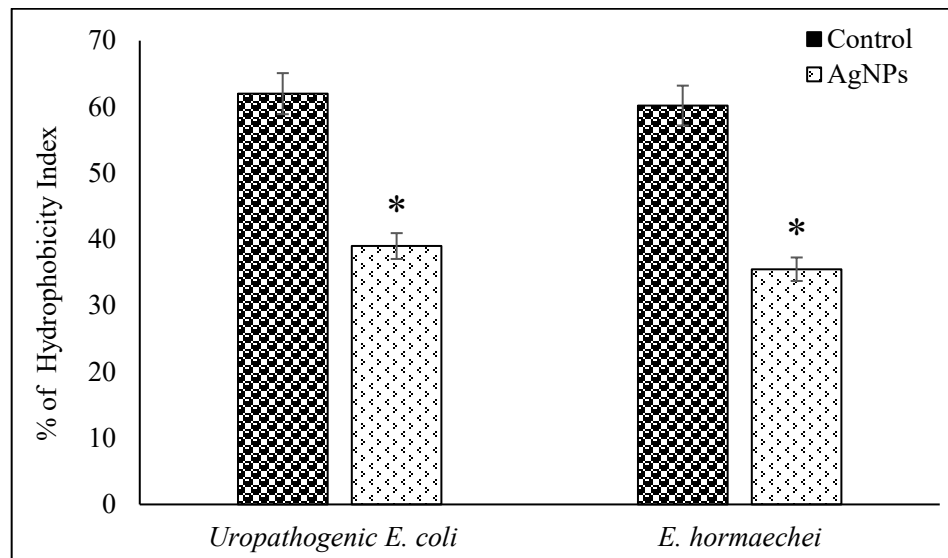


Fig. 8(A, B)

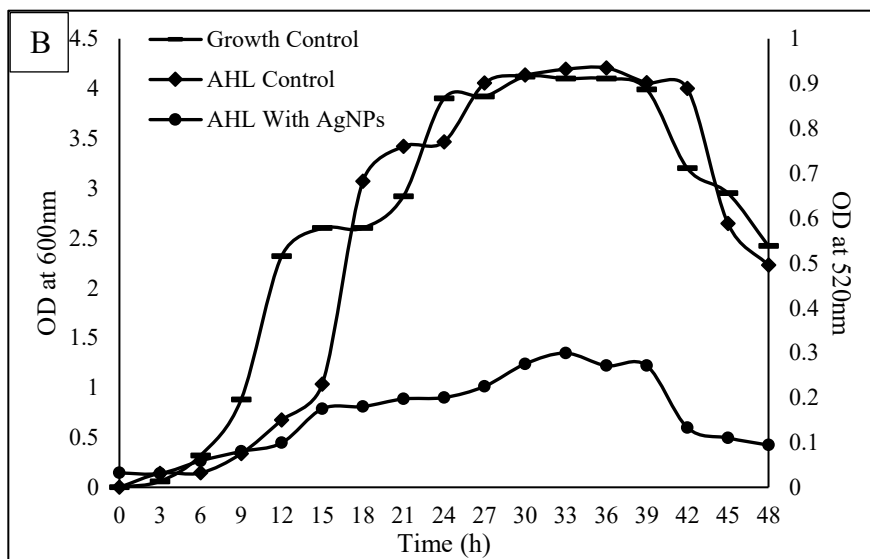
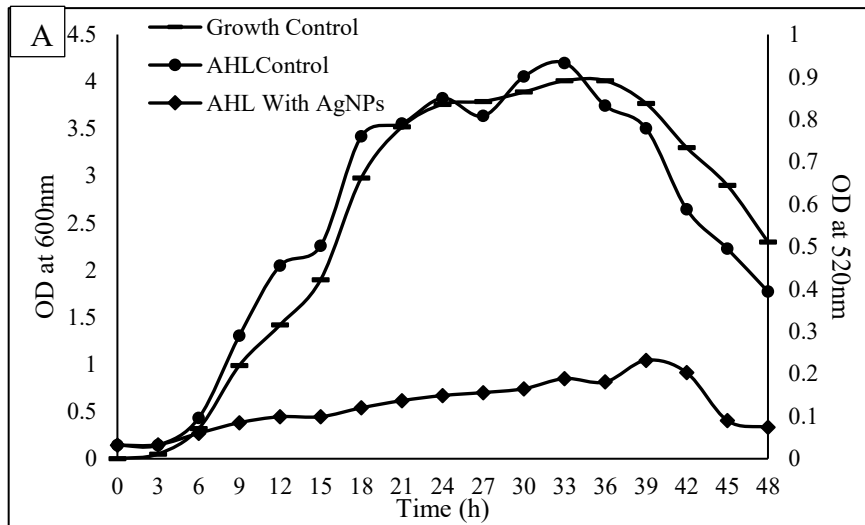


Fig. 9(a-d)

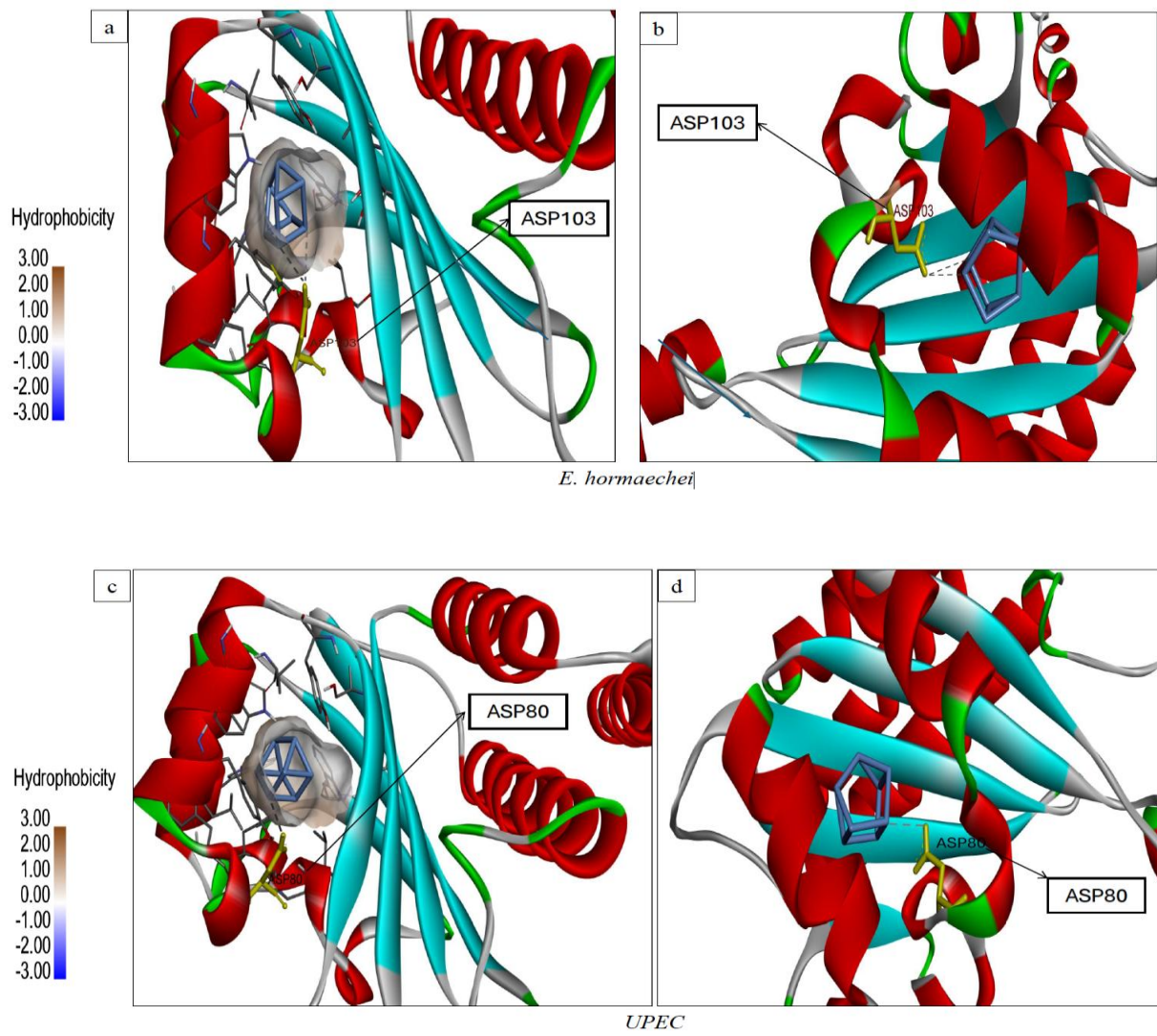


Fig. 10(A)

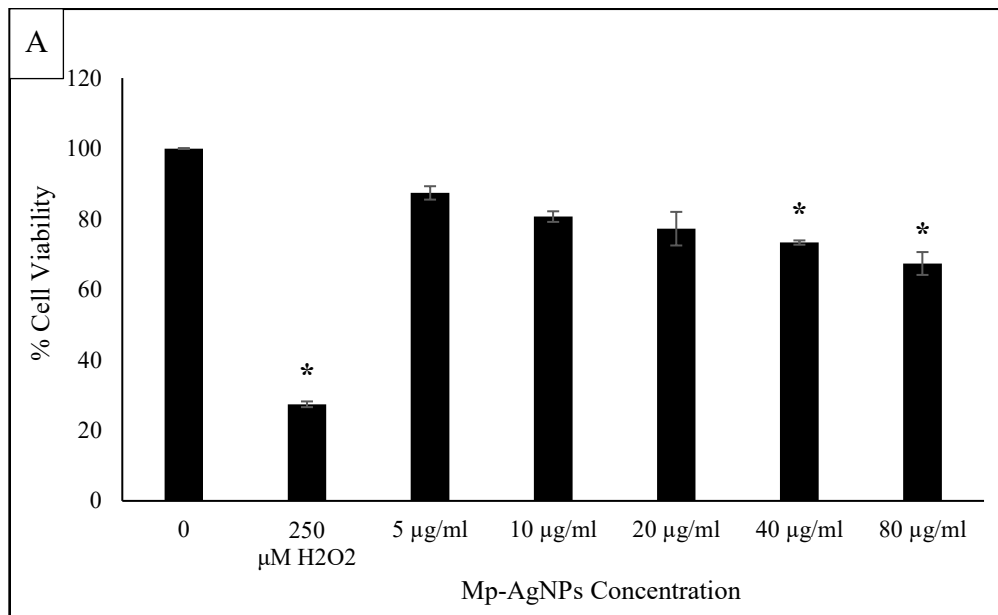
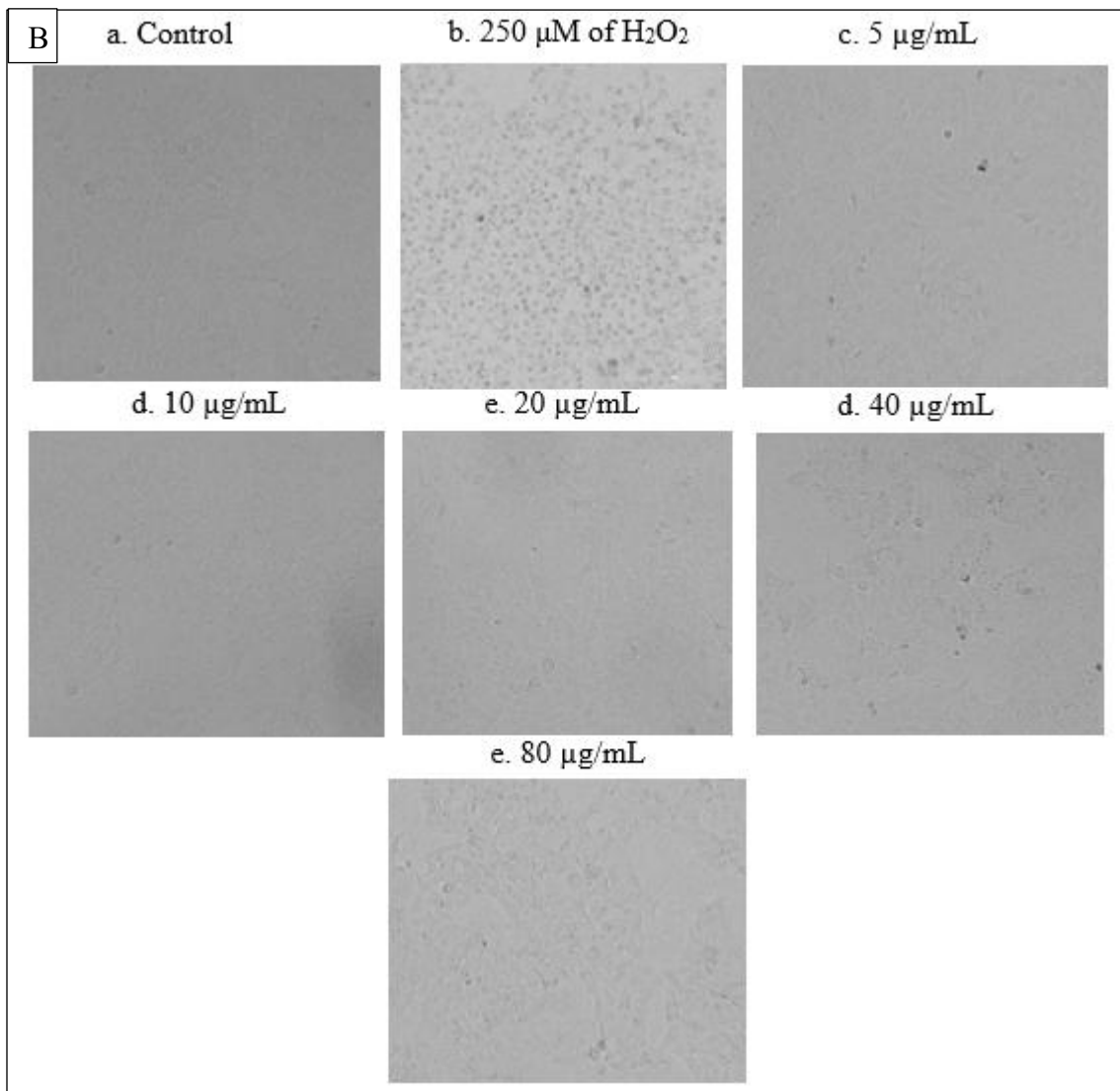


Fig. 10(B)



Supplementary Files

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- [SupplementaryDataWJMB.docx](#)