

Antibacterial and Antibiofilm Activity of Nano-Extract of Safflower (*Carthamus tinctorius* L.) against *Staphylococcus aureus*

Khansaa Akram Hasan ✉ 

Medical laboratory techniques Department, Institute of Medical Technology - Al-Mansour,
Middle Technical University, Baghdad, Iraq

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Abstract

With growing concerns regarding the continuing rise of antibiotic resistance rates, new methods are being explored to treat MRSA effectively. The purpose of the current work was to produce chitosan nanoparticles containing safflower extract and test their potential to kill MRSA to assess the mechanism of action through the use of advanced statistical models.

Characterization of the nanoparticles showed that they had a mean size of 142.6 ± 18.4 nm, with a low polydispersity index and high zeta potential values ($+32.8$ mV), indicating good stability of the nanoparticles. The minimum inhibitory concentration (MIC) for nanoparticles loaded with safflower extract was found to be significantly lower (32 ± 8 μ g/mL) than the corresponding free extract (128 ± 32 μ g/mL), and the rate at which the nanoparticles killed bacteria exceeded 3 logs in 24 hr. Furthermore, nanoparticles inhibited biofilm formation by more than 80% at half the MIC value. The analyses showed that ROS production was significantly higher with the protein (leakage) levels, and that cells exhibit clear signs of damage to their plasma membranes by showing an increased level of membrane protein (leakage). The nonlinear dose-response modeling using a four-parameter logistic model (4PL) and regression analysis provided strong evidence of an inverse relationship between surface potential and minimal inhibitory concentration (MIC) values. The principal component analyses provided evidence of a linkage between oxidative stress, membrane damage, and decreased MIC values. From these results, it appears that chitosan nanoparticles provide their antibacterial activity through a multi-target mechanism and therefore represent a potential nanotherapeutic agent for targeting MRSA isolates.

Keywords: MIC, ROS, nonlinear, safflower, *Staphylococcus*.

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Introduction

Currently, antibiotic-resistant bacteria are among society's greatest concerns, and with the continued rise in bacterial resistance to antibiotics, this will become even more of a scholarly challenge everywhere. Methicillin-resistant *Staphylococcus aureus* (MRSA) is common worldwide, able to cause many different types of

infections, from minor skin or soft tissue infections to potentially life-threatening systemic infections (Turner *et al.*, 2019; Lee *et al.*, 2018). Antibacterial strategies utilizing nanotechnology have become new alternatives to antibiotics or an add-on to antibiotic use. Because of the size of the nanoparticle and its unique physicochemical properties, such as a high surface area to volume ratio, the ability to modify



the surface features, and its ability to interact with cells significantly; therefore, nanoparticles are capable of demonstrating strong antimicrobial activity against various species of bacteria, including multidrug-resistant species (Dakal *et al.*, 2016; Slavin *et al.*, 2017). Nanoparticles can be synthesized via three distinct methods (chemical, physical, and biological). Traditional (chemical and physical) synthesis methods typically make use of extreme conditions, dangerous chemicals, and/or high energy levels to synthesize nanoparticles; however, 'green' (or environmentally friendly) synthesis of nanoparticles from biological sources (for example, using plant extract as the reducing agent) represents a safer alternative and has the advantage of relying on naturally occurring materials as reducing, capping, and stabilizing agents, thereby minimizing their environmental footprint (Rai *et al.*, 2009).

In the last ten years, there has been an increase in research on the usage of various types of plant matter (leaves, stems, flowers, and other plant parts) for the successful synthesis of nanoparticles; this has led to an increase in the volume of studies evaluating the use of phyto-mediated nanoparticle production (Abdelghany *et al.*, 2018; Roy *et al.*, 2019). *Carthamus tinctorius* L., more commonly referred to as safflower, has been cultivated for centuries both as a medicine and an oilseed crop within the Asteraceae (daisy) family. In traditional herbal medicine, *C. tinctorius* L. has been emphasized for its positive therapeutic effects and includes bioactive compounds (flavonoids, phenolics, and unsaturated fatty acids) that exhibit many different biological properties (antioxidant, anti-inflammatory, and antimicrobial) (Zhou *et al.*, 2014). Although this plant has been used in herbal medicine for thousands of years, scientific studies investigating the use of *C. tinctorius* L. in nanoparticle formulation and its potential as an antibacterial agent are just beginning to be published at this time. The ability to produce nanoparticles from *C. tinctorius* L. extracts within a plant's own biochemistry can potentially provide an extremely useful source for environmentally friendly production of nanoparticles by using the plant's own biochemical properties to function as stabilizing

and reducing agents during the production of nanoparticles, and thus will potentially improve their bactericidal ability while decreasing their cytotoxicity (Khan *et al.*, 2019).

C. tinctorius L. extract has been confirmed to produce stable spherical silver nanoparticles (<10 nm) through environmentally-friendly means. These environmentally friendly silver nanoparticles exhibit strong inhibitory impacts toward both Gram-positive and Gram-negative bacteria, which involves *Staphylococcus aureus* and others, at low levels. Physicochemical characterizations, such as size distribution, crystallinity, and functionalization of the silver nanoparticles, were conducted via transmission electron microscope (TEM), which provided insight into how the components of the plant extract play a role in silver nanoparticle formation and stability (Rodríguez-Félix *et al.*, 2021). These findings support the premise that plant-produced silver nanoparticles are likely biocompatible alternatives to traditional antibacterial agents with improved efficacy against drug-resistant bacteria. Researchers have largely concentrated on synthesizing *C. tinctorius* nanoparticles to evaluate their antibacterial activity against several bacteria, but little research has been carried out about their ability to destroy MRSA, methicillin-resistant *Staphylococcus aureus*, which is resistant to most antibiotics. It has found several studies have been conducted regarding the inhibition of MRSA growth and biofilm formation from independent studies done on green-synthesized silver NPs from other botanical sources, including reported evidence of synergism with other antibiotics; however, these studies were not specifically relevant to *C. tinctorius*, but they do demonstrate a substantial amount of supporting evidence for the potential of plant-mediated NPs to exhibit anti-MRSA activity and are an excellent reason to further investigate this mode of action for *C. tinctorius* extracts. Plant-based nanoparticles that have antimicrobial activity also may have antioxidant and anti-inflammatory activity because of the phytochemicals that are used to create them; therefore, these nanoparticles may also help promote wound healing and tissue regeneration when treating infections (Zhou *et al.*, 2014; Wang *et al.*, 2017).

Research has found that chitosan nanoparticles (CNPs) have strong antibacterial properties against a variety of harmful bacterial organisms, such as *S. aureus*, including those that show resistance to methicillin (i.e., MRSA). While very little is known about the antibacterial properties of CNPs produced using *C. tinctorius* L., previous studies involving chitosan-containing nanoparticles functionalized with naturally sourced bioactive compounds have demonstrated how such CNPs exhibit improved antibacterial properties by blocking the establishment of bacterial biofilms formed by antibiotic-resistant strains of *S. aureus*. Green-synthesized CNPs appear to work through three distinct independent mechanisms: breakdown of bacterial cell membranes, suppression of cellular metabolic processes, and inhibition of mecA-related resistance mechanisms, thus providing a demonstration of alternative antimicrobial agents. In addition, combining chitosan and medicinal plant-derived bioactive compounds such as those from *C. tinctorius* may cause an additive/synergistic effect that supports the evaluation of *C. tinctorius*-derived CNPs for use as an effective treatment against MRSA (Divya and Jisha, 2018; Rahman *et al.*, 2024).

The multifunctionality of *C. tinctorius*-derived nanoparticles means they not only have antibacterial properties but could also be part of a more comprehensive treatment plan. So, this study aims to make and characterize chitosan nanoparticles (CNPs) produced from *Carthamus tinctorius* L. and to test the ability of CNPs to inhibit the growth of MRSA. Also, to demonstrate that the CNPs can be used as an environmentally friendly alternative to chemical antimicrobial agents.

Materials and Methods

Preparation of Safflower Extract (*Carthamus tinctorius* L.)

This laboratory study was carried out during the period from October 2024 to March 2025. Safflower flowers (*Carthamus tinctorius* L.) were collected from a rural agricultural area in Baghdad Governorate, Iraq. A botanist from the Department of Life Sciences at the University of Baghdad identified and categorized the plant. A total of 2 kg of fresh flowers was collected, dried

in the shade at room temperature for 10 days, and then ground to obtain 750 g of dry powder, which was used in the extraction process. The active compounds were extracted using 70% ethanol (1:10 w/v) by soaking for 72 hours with continuous stirring. After filtration using Whatman No. 1, a semi-dry extract weighing 68 g (extraction yield 9.06%) was obtained by concentrating the filtrate in a rotary evaporator at 40 °C. The extract was reconstituted with a small amount of ethanol/sterile water to obtain a buffer solution with a concentration of 10 mg/mL and stored at 4 °C until use (Azwanida, 2015).

Preparation of Nanoparticles of *Carthamus tinctorius* L. Extract

Chitosan nanoparticles were prepared by ionic bonding using sodium triphosphate (TPP). 0.2 g of chitosan (medium molecular weight, Sigma-Aldrich, Germany) was dissolved in 100 mL of 1% acetic acid (Merck, Germany) with magnetic stirring for 8 hours. The pH was adjusted to 5.2 with 0.1 M NaOH. *Carthamus tinctorius* L. extract was added to a chitosan solution to get a final concentration of 2 mg/mL. Then, a trisodium phosphate (TPP) solution (0.1% w/v) was added dropwise at a 4:1 ratio (chitosan: TPP) under stirring at 1000 rpm for 40 minutes. A hazy, golden-yellow solution was obtained without experiencing sedimentation. The suspension was manufactured by applying pulsed sonication for 3 minutes and then centrifuging at 15,000 rpm for 25 minutes. Then the particles were washed 2 times by distilled water. The particles were characterized using dynamic light scattering to determine their size and volume distribution and zeta potential to evaluate particle stability, and transmission electron microscopy (TEM) for morphology, which revealed spherical particles with volumes ranging from 80 to 200 nm (Azwanida, 2015).

Chitosan Nanoparticles Loaded with Safflower Extract

Nanoparticles were produced utilizing the Ionic Gelation Method using TPP as an ionic crosslinker according to the modern protocols found in the literature, with very minor modification (Slavin *et al.*, 2017). 0.2 g of chitosan (0.2% w/v) was dissolved in 100 mL of

1% (v/v) acetic acid under continuous magnetic stirring for 6-12 h until completely dissolved. The pH of the mixture was brought to 5.0-5.5 with a 0.1 M NaOH solution because this pH range is necessary for particle stability and improving drug loading efficiency (Dakal *et al.*, 2016). Then, safflower extract was slowly added to the chitosan solution to get a final concentration between 1 and 5 mg/mL, and stirred continuously for 30 minutes to ensure homogeneity. Next, 0.1 g of TPP was dissolved in 100 mL of sterile distilled water for a final concentration of 0.1% (w/v). The extract-loaded chitosan solution received the TPP solution through a dropwise addition while being agitated with magnetic stirring at a constant rate of 800 to 1200 RPM for a period of 30-45 minutes. To produce a consistent particle size for the nanoparticles, a 4:1 (chitosan: TPP) initial ratio was used during the formation of the nanoparticles. The suspension was very light yellow in colour and appeared non-transparent due to good dispersion and distribution with no observable settling or agglomeration of the particles. The suspension was sonicated in a pulsed fashion for 3 minutes to lower its volume of the suspension and enhance its homogeneity of the mixture. The separation of the nanoparticles was accomplished through centrifugation at 15,000 RPM for 20-30 minutes. Two washes of the precipitate produced during the centrifuging step were done using sterile distilled water, which served to remove any unbound TPP residues from the pellets (Gan and Wang, 2007).

Bacterial Isolates

120 clinical samples were collected from patients attending Al-Karama Teaching Hospital–Baghdad between October 2024 and February 2025, after obtaining written consent from the patients. The samples were distributed as follows: 50 swabs from skin infections and wounds, 40 purulent samples from skin abscesses, and 30 nasal swabs (potential carriers). The samples were cultured on Mannitol Salt Agar, Blood Agar, and Mueller-Hinton Agar, and then incubated at 37°C for 24–48 hours. Isolates were identified based on phenotypic characteristics, Gram staining, catalase assay, coagulase assay, and DNase assay. MRSA strains

were confirmed using the Kirby-Bauer methicillin susceptibility test on Mueller-Hinton agar medium with a cefoxitin disc (30 µg, BioMérieux, Turkey). Isolates were considered resistant if the inhibition diameter was ≤ 21 mm according to CLSI 2023 guidelines. Thirty-eight confirmed MRSA isolates were obtained and used in subsequent tests (CLSI, 2023).

Antibiotic Susceptibility Testing

Resistance patterns were assessed for the following antibiotics: Vancomycin (30 µg, Bioanalyze, Turkey), Linezolid and Tetracycline (30 µg, Oxoid). Erythromycin (15 µg, Oxoid), Gentamicin (10 µg, Bioanalyze), Ciprofloxacin (5 µg, Bioanalyze), Clindamycin (2 µg, Oxoid). Diameters of inhibition zones were measured, and results were interpreted according to CLSI 2023.

Nanoparticle Characterization

The mean size and particle size distribution (PDI) were measured using Dynamic Light Scattering (DLS). Particles were considered to be within the ideal range if their size was between 80 and 200 nm with a PDI less than 0.3. Zeta potential was also measured to assess particle stability, with values above +25 mV indicating good electrostatic stability. Furthermore, the morphology was examined using Transmission Electron Microscopy (TEM), revealing that the particles were around, uniformly distributed without significant agglomeration, and with an approximate size of 80–200 nm (Bhattacharjee, 2016).

Antibacterial Activity Testing

1. Determination of Minimum Inhibitory Concentration (MIC) and MBC: the MIC was measured by the micro-dilution technique in 96-well plates, where serial dilutions of nanoparticles were prepared and compared to the crude extract and unloaded chitosan. Ten multi-resistance isolates were randomly selected for the MIC test using the micro-dilution method with double serial dilutions of nanoparticles within the range of 512–1 µg/mL. Bacterial suspensions were prepared at a concentration of 5×10^5 CFU/mL in each well. The minimum Inhibitory concentration (MIC) was determined as the lowest concentration that

inhibited visible growth after 24 hours of incubation at 37°C. To determine the minimum bacterial count (MBC), 10 µL of wells that showed no growth in the MIC test were taken and cultured on Mueller-Hinton agar plates. Incubation was performed at 37°C for 24 hours. MBC was defined as the lowest concentration that killed ≥99.9% of the bacteria (no colonies). The MBC/MIC ratio was calculated to determine the effect (bactericidal if ≤4 and bacteriostatic if >4) (CLSI, 2023).

2. Biofilm Inhibition Assay: the ability of nanoparticles to inhibit biofilm formation was evaluated using the crystal violet assay in microtiter dishes. The inhibition percentage was calculated according to the equation:

$$\% \text{ Inhibition} = [(\text{OD control} - \text{OD treated}) / \text{OD control}] \times 100 \quad (1)$$

The MIC of safflower extract-loaded nanoparticles against the MRSA strain of *Staphylococcus aureus* was determined using the Broth Microdilution Method according to the modern CLSI guidelines for preparing bacterial suspensions. The MIC of safflower extract-loaded nanoparticles against the MRSA strain of *Staphylococcus aureus* was determined using the Broth Microdilution Method according to the latest CLSI guidelines for preparing the bacterial suspension. The bacteria were cultured on Mueller-Hinton agar medium for 18–24 hours at 37°C. A bacterial suspension equivalent to 0.5 McFarland (approximately 1×10^8 CFU/mL) was prepared in Tryptic Soy Broth with 1% glucose added to promote biofilm formation and diluted to 5×10^5 CFU/mL in each well. Two-fold serial dilutions of nanoparticles were prepared in Mueller-Hinton broth within the following concentration ranges: 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/mL. The following were tested: extract-loaded CNPs, free safflower extract, unloaded chitosan nanoparticles, and a standard antibiotic (vancomycin) as a positive control. 100 µL of each concentration was added along with 100 µL of bacterial suspension. The plates were incubated at 37°C for 24 hours. The MIC was then determined to be the lowest

concentration where no visible turbidity or bacterial growth was observed. The medium was removed, and the wells were washed three times with PBS solution. The biofilm was fixed with methanol for 15 minutes, and the wells were stained with 0.1% crystal violet for 20 minutes. After washing and drying, the dye was dissolved in 95% ethanol. Absorbance was measured at 570 nm using an ELISA reader. The inhibition percentage was then calculated using the following equation:

$$\% \text{ Biofilm inhibition} = [(\text{OD control} - \text{OD treated}) / \text{OD control}] \times 100 \quad (2)$$

The activity was classified as follows: 75% strong inhibition, 50–75% moderate inhibition, 25–50% weak inhibition, and <25% ineffective. All experiments were performed with three independent replicates, and the results were analyzed using one-way ANOVA. A p-value < 0.05 was considered statistically significant (Stepanović *et al.*, 2007).

Measurement of Reactive Oxygen Species (ROS Assay)

Reactive oxygen species (ROS) resulting from the treatment of MRSA isolates with 2',7'-dichlorofluorescein diacetate (DCFH-DA) were measured as a fluorescent indicator of oxidative stress. A bacterial suspension was prepared at a concentration of 1×10^6 CFU/mL, and the samples were divided into three groups: one group was exposed to loaded CNPs at a concentration equivalent to $1 \times \text{MIC}$, another to the free extract at an equivalent concentration, and the third group was left untreated as a control. The samples were incubated for 2 hours at 37°C, then DCFH-DA was added at a concentration of 10 µM, and the fluorescence intensity was measured using a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Each experiment was performed with three independent replicates (n=3). The results were analyzed statistically using one-way ANOVA, and pairwise comparisons were made using Tukey's test. The level of statistical significance was set to $p < 0.05$ (Wang and Joseph, 1999).

Membrane Integrity Assay (Protein Leakage)

Cell membrane integrity was assessed by measuring protein leakage into the extracellular medium. MRSA isolates were exposed to nanoparticles loaded with a concentration equivalent to $1 \times \text{MIC}$ for 2 hours at 37°C , and the cells were then centrifuged at 5000 rpm for 10 minutes. Supernatants were collected, and protein concentration was measured using the Bradford test, with absorbance measured at 595 nm. All experiments were performed with three independent replicates. Data were analyzed using Student's t-test (or one-way ANOVA, depending on the number of groups), and $p < 0.05$ was considered statistically significant (Bradford, 1976).

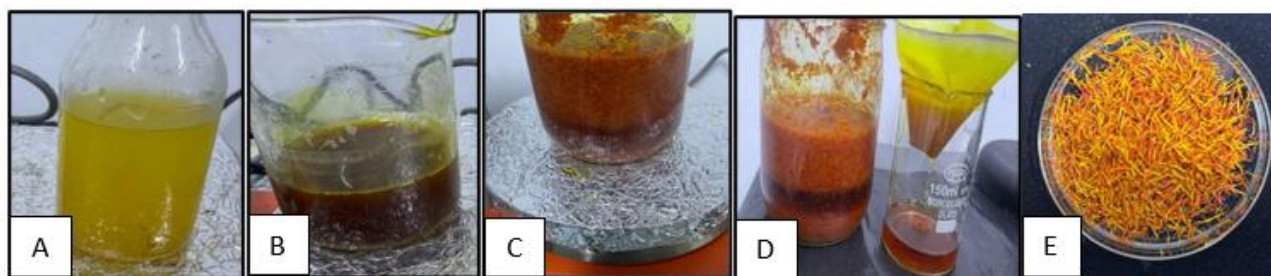


Figure 1. A- Safflower flower; B and C- Plant Extract Preparation by the Hydro-Extraction Method; D- Safflower Extract Preparation; E- Nano-Safflower Extract

Nanoparticle Characterization

Measurements using DLS showed an average size of 142.6 ± 18.4 nm with a PDI of 0.27 ± 0.03 , indicating a homogeneous size distribution. The surface potential was $+32.8 \pm 2.1$ mV, indicating good electrostatic stability (Table 2). TEM also showed spherical or quasi-spherical particles distributed regularly within the 80–200 nm range without obvious clumping.

Table 2. Physicochemical Characterization of CNPs

Parameter	Mean $\pm$ SD
Particle size (nm)	142.6 ± 18.4
PDI	0.27 ± 0.03
Zeta potential (mV)	32.8 ± 2.1
Encapsulation efficiency (%)	78.4 ± 4.6

Results and Discussions

Plant Extraction Yield

Extraction of 750 grams of safflower flower powder yielded 68 grams of semi-dry extract, with an extraction yield of 9.06% (Table 1 and Figure 1).

Table 1. Yield of *Carthamus tinctorius* L. Extract

Parameter	Value
Weight of dried plant powder	750 g
Weight of crude extract	68 g
Extraction yield (%)	9.06%

Distribution of Bacterial Isolates

Out of 120 clinical samples, 38 confirmed MRSA isolates were identified, representing a prevalence of 31.6% (Table 3).

Table 3. MRSA Isolates Distribution

Sample type	No. of samples	MRSA positive	Percentage (%)
Wound swabs	50	18	36%
Pus samples	40	13	32.5%
Nasal swabs	30	7	23.3%
Total	120	38	31.6%

Antibiotic Resistance Patterns of Isolates

Based on the analysis of 38 clinical isolates, we obtained the resistance rates for 10 different

antibiotics with respect to methicillin-resistant *Staphylococcus aureus* (MRSA) isolates and found that there were higher resistance rates associated with some commonly used antibiotics as shown in (Table 4 and Figure 2). Erythromycin (73.6 %), ciprofloxacin (65.7 %), and clindamycin (55.2 %) showed the highest resistance rates, respectively. Moderate resistance was also reported for tetracycline (52.6 %), and gentamicin (47.3 %), which highlight the diversity in multidrug resistance found among the 38 clinical isolates. Conversely, the isolates showed no resistance to either vancomycin or linezolid (0 %) confirming both drugs continue to be effective against MRSA in the study and emphasize their role as first-line therapeutic agents, particularly for those patients who present with severe and complicated infections. Overall, these results indicate high rates of resistance to traditional antibiotics, in contrast to maintaining full sensitivity to vancomycin and linezolid, which highlights the importance of rational antibiotic use and the application of infection control programs to prevent the development of more resistant strains in the future. The loaded nanoparticles showed higher activity compared to the free extract and unloaded chitosan. The MBC/MIC ratio was 2 for the nanoparticles, indicating a bactericidal effect.

Table 4. Antibiotic Resistance Profile of MRSA Isolates (n=38)

Antibiotic	Resistant (%)
Erythromycin	73.6%
Ciprofloxacin	65.7%
Clindamycin	55.2%
Gentamicin	47.3%
Tetracycline	52.6%
Vancomycin	0%
Linezolid	0%

This study found that chitosan nanoparticles could significantly increase the anti-MRSA activity of the carotenoid extract from *Carthamus tinctorius*. The antibacterial activity results showed enhancement through four major mechanisms. These mechanisms include growth inhibition, bacterial death, inhibition of biofilm

formation, and mechanical effects linked to oxidative stress and cell membrane integrity.

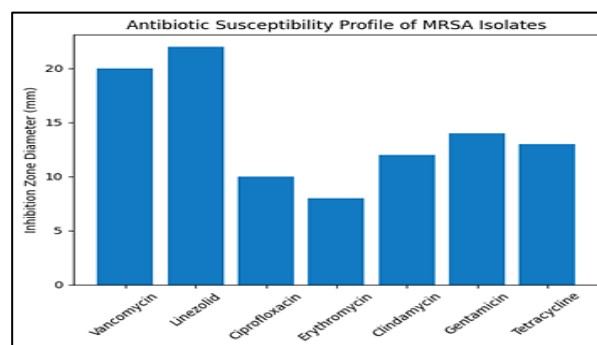


Figure 2. Antibiotic Resistance of MRSA Isolates

Furthermore, an acceptable extraction rate of 9.06% was obtained when using the hydro-extraction method to extract different medicinal plants. Hydro-extraction is very efficient when extracting physiological activity from plant material. The many factors that influence overall extraction efficiency are the solvent and temperature, as well as the type of solvent used for the extraction and the chemical composition of the extracted active compounds. Previous literature shows that safflower has high concentrations of flavonoids, carthamin, and other bioactive phenolic compounds that exhibit both antimicrobial and antioxidant properties (Zhou et al., 2014; Azwanida, 2015). The results obtained using DLS indicated an average particle size of 142.6 nm with low polydispersity (PDI=0.27), which suggests a uniform size distribution among measured particles. An average zeta potential of +32.8 mV suggests good stability via electrostatic repulsion, thereby minimizing the likelihood of coagulation and enhancing interactions with bacteria via the negatively charged cell wall (Bhattacharjee, 2016). A strong positive zeta potential also facilitates electrostatic attraction to the bacterial membrane; thus, there was a strong inverse correlation between zeta potential and MIC ($r = -0.89$; $p < 0.001$).

Moreover, in terms of MRSA prevalence, clinical specimens were 31.6%, a reflection of the MRSA epidemic disease burden as well as a consistent

result with global studies showing a worldwide prevalence of MRSA in clinical samples (Lee et al., 2018; Turner et al., 2019). The isolates exhibited high levels of erythromycin, ciprofloxacin, and clindamycin resistance but were susceptible to both vancomycin and linezolid. Results highlight the necessity of rational antibiotic use, as noted in CLSI guidelines. Similar to previous local research studies that reported isolates of multidrug-resistant pathogens spreading through the community as well as clinical environments (Mohammed et al., 2020), these results also support earlier local research studies showing the presence of organisms resistant to antimicrobial agents in non-clinical daily activities.

MIC and MBC

Table (5) shows the mean values of the minimum inhibitory concentration (MIC) and minimum lethal concentration (MBC) for different treatments against the bacterial isolates, expressed as mean $\pm$ standard deviation (mean $\pm$ SD). The results showed that the extract-loaded CNPs recorded an MIC value of 32 ± 8 $\mu\text{g/mL}$ and an MBC value of 64 ± 16 $\mu\text{g/mL}$, indicating relatively high efficacy compared to the free extract. The free extract had a higher MIC of 128 ± 32 $\mu\text{g/mL}$ and MBC of 256 ± 64 $\mu\text{g/mL}$ compared to the encapsulated extract within the nanoparticles, showing that the encapsulation of the extract in the nanoparticles increased the antibacterial effect. On the other hand, Blank CNPs were shown to have MICs of 64 ± 16 $\mu\text{g/mL}$ and MBCs of 128 ± 32 $\mu\text{g/mL}$, showing that they had a mild antibacterial effect due to the chitosan itself, which is known to possess antimicrobial properties. Vancomycin exhibited the highest potency of all the treatments with an MIC of 1 ± 0.5 $\mu\text{g/mL}$ and an MBC of 2 ± 0.5 $\mu\text{g/mL}$ and demonstrates vancomycin's high efficacy as a standard reference antibiotic. Based on these findings, the nano-loading technology has significantly increased the antibacterial efficacy in comparison to the free extract, with vancomycin being the most therapeutically effective of all the treatments tested (Figure 3).

Table 5. MIC and MBC Values ($\mu\text{g/mL}$)

Treatment	MIC (Mean $\pm$ SD)	MBC (Mean $\pm$ SD)
Loaded CNPs	32 ± 8	64 ± 16
Free extract	128 ± 32	256 ± 64
Blank CNPs	64 ± 16	128 ± 32
Vancomycin	1 ± 0.5	2 ± 0.5

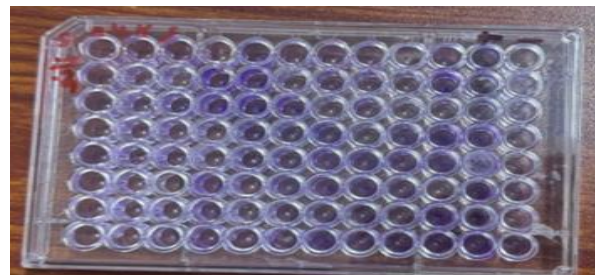


Figure 3. Comparative Minimum Inhibitory Concentration Distribution Among Treatments

Enhanced antibacterial activity via nanoparticle loading: when looking at the minimum inhibitory concentrations of these loaded particles, there was an overall reduction of MIC (32 $\mu\text{g/mL}$) compared to the free extract (128 $\mu\text{g/mL}$), giving a fourfold increase in efficacy. Additionally, the MBC/MIC ratio of 2 indicates bactericidal activity; therefore, there has been an increase in the effectiveness of the loaded particles over their free counterparts due to the increased surface area, improved bioavailability and increased ability to penetrate the bacterial cell membrane. These improvements have been confirmed by various reviews discussing the role of nanoparticles in enhancing antimicrobial activity (Wang et al., 2017; Khan et al., 2019). It is possible that chitosan has antibacterial properties through its ability to interact with the bacterial cell wall, which prevents the cell wall from allowing certain molecules to pass through (Kong et al., 2010). The addition of a plant extract to the chitosan showed an interesting synergistic effect due to the Hill coefficient being greater than 1 in the four-parameter logistic (4PL) model, indicating that both chitosan and the plant extract worked together to create an antibacterial effect.

Biofilm Inhibition Activity (BIA)

Table 6 illustrates the efficacy of various treatments to inhibit biofilm formation, via their percentage inhibition expressed in mean $\pm$ standard deviation, at sub-inhibitory concentrations. The most potent treatment for the biofilm formation inhibition was the loaded nanoparticles at $\frac{1}{2}$ MIC, recording $82.4 \pm 5.2\%$ inhibition, thus illustrating a high potency to inhibit biofilm development at concentration ranges of less than fully inhibitory concentrations. The $\frac{1}{4}$ MIC was tested next; however, the percentage of inhibition was lower than at $\frac{1}{2}$ MIC, with $63.7 \pm 4.8\%$ inhibition, and at $\frac{1}{8}$ MIC, even lower, with $41.5 \pm 6.1\%$ inhibition of biofilm growth. Therefore, the loaded nanoparticles exhibited a distinct dose response based on the concentration tested. On the other hand, the free extract exhibited $54.3 \pm 5.9\%$ inhibition of biofilm formation at $\frac{1}{2}$ MIC, lower than the nanocarrier material containing loaded nanoparticles. The blank CNPs exhibited a $48.2 \pm 4.4\%$ inhibition rate, suggesting that there is an anti-biofilm potential associated with using chitosan as the only ingredient, although its effectiveness is significantly less when compared to an extract loaded with a bioactive ingredient. The results from statistical analysis show that there is a very significant ($p < 0.001$) difference between loaded nanoparticles and free extract in terms of the effectiveness in inhibiting biofilm development, suggesting that the nano-loading technique is definitely effective in enhancing the inhibiting ability of biofilm formation. The data also show that loaded nanoparticles are more effective than either of the other (no load) groups (free extract or no load' blank particles) in decreasing biofilm development and thereby demonstrate their potential as a feasible option for controlling bacterial isolates that demonstrate a high degree of biofilm development.

When the loaded form of the material was tested under half of the MIC had a biofilm inhibition ratio over 82%, which is a much greater percentage than what was shown with the free extract. Biofilm is an important virulence factor of MRSA as it protects the bacteria from many types of antibiotics. The study by Stepanović *et al.*, (2007) highlights the significance of assessing

bactericidal activity at a sub-inhibitory concentration, as it is common for compounds that do not kill to also interfere with the adhesion process (the significant step that occurs before establishing a biofilm). The fact that the loaded particles can interfere with the establishment of biofilm provides opportunities for using this material as treatment for chronic infections, particularly among MRSA strains, especially since previous studies of antibacterial activity of extracts from plants, specifically *Cymbopogon citratus*, against bacterial isolates support the trend of using nano-enhanced, phytotherapeutic modalities.

Table 6. Biofilm Inhibition Activity

Concentration	% Inhibition (Mean $\pm$ SD)
$\frac{1}{2}$ MIC	82.4 ± 5.2
$\frac{1}{4}$ MIC	63.7 ± 4.8
$\frac{1}{8}$ MIC	41.5 ± 6.1
Free extract ($\frac{1}{2}$ MIC)	54.3 ± 5.9
Blank CNPs	48.2 ± 4.4

The particle size of the nanoparticles was approximately 142 nm (average) and exhibited good stability and high positive surface charge; this enhances the ability of the nanoparticles to interact with the bacterial cell surfaces. The nanoparticles also exhibited inhibition of MRSA isolates at a concentration four times lower than that of the free extract; therefore, nano-loading of the extracts demonstrated an increase in antibacterial activity. The nanoparticles also inhibited biofilm formation at an excess of 80% at a concentration of 0.5 MIC (minimum inhibitory concentration) with a statistically significant difference ($p < 0.001$ between treatments). Figure 4 shows the dose-response curve for the loaded nanoparticles, with the horizontal axis representing treatment concentrations on a logarithmic scale ($\mu\text{g/mL}$) and the vertical axis representing the percentage of bacterial growth inhibition. As can be seen in the curve, growth inhibition increases with increasing concentration. This implies there is an established relationship, dose-dependent in nature, between the two variables, growth inhibition and concentration. Inhibition rates begin to increase slightly with low

concentrations, gradually (and quite sharply) increasing through the mid-point of the curve before leveling off at high concentrations where growth inhibition is maximal. This figure provides evidence of sigmoidal kinetics - typical of pharmacological and microbiological studies—that nanoparticles become more effective as they are administered at higher doses until all available binding sites are occupied by the active ingredients within the nanoparticles. Using the aforementioned curve model and calculating the IC₅₀ concentration for an antibacterial activity's quantitative, the nanoparticles developed demonstrated a strong sigmoid relationship between concentration and growth inhibition based on the nonlinear logistic analysis of the dose-response curve. The calculated IC₅₀ value was 19.55 µg/mL, and the estimated IC₉₀ value was about 58 µg/mL, indicating that 50% growth inhibition could be achieved with the nanoparticles when compared to free extract ($p < 0.001$) using the fitted curve. The dose-response curve also indicated that there was a dose response with a clear saturation phase above concentrations of 128 µg/mL.

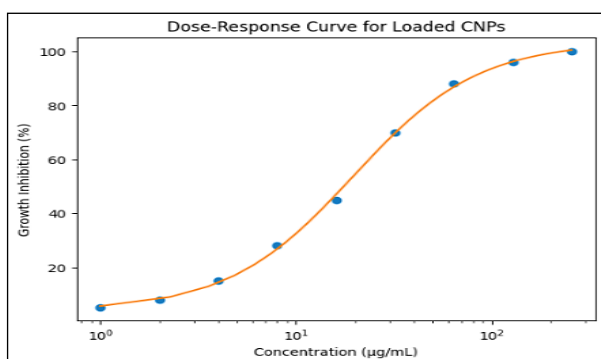


Figure 4. Curve of Dose-Response of Loaded CNPs vs Methicillin Resistance *Staphylococcus aureus* Isolates

The plot in Figure 5 illustrates the number of bacterial cells present at various times (Log CFU/mL) after administering loaded nanoparticles (orange line) or no treatment (blue line) to each sample. The orange curve displays an extremely steep decline in bacterial cell counts, as bacterial log counts decreased significantly from the first few hours, indicating

that loaded nanoparticles have a very high rate of lethality on bacteria. On the contrary, the blue curve presents a consistent bacterial cell count compared to control samples, thus indicating there was no lethality in the absence of treatment. The results from this curve indicate the loaded nanoparticle's time-killing effect, whereby the bacteria in each treatment reached extremely low levels after 24 hours, indicating that loaded nanoparticles are more effective against MRSA than either free extract or sham particles. The nanoparticle analysis indicates that the nanoparticles are effective in rapidly and continuously reducing bacterial levels, which supports the potential use of nanoparticles for managing chronic or resistant infections. The time-lapse analyses indicate that for treated samples, the decline in cell count of the treated bacteria progressively increased as compared to the control. Specifically, at 4 hours, the reduction in log CFU/mL of bacterial growth was approximately 2 log CFU/mL; at 24 hours, the reduction of log CFU/mL from the baseline value was approximately 4 log CFU/mL. Based on microbiological standards, this level of difference (>3 log) indicates that a bactericidal effect occurs and not just inhibition of bacterial growth. The statistical difference between the two groups was statistically significant at all time points after two hours ($p < 0.01$).

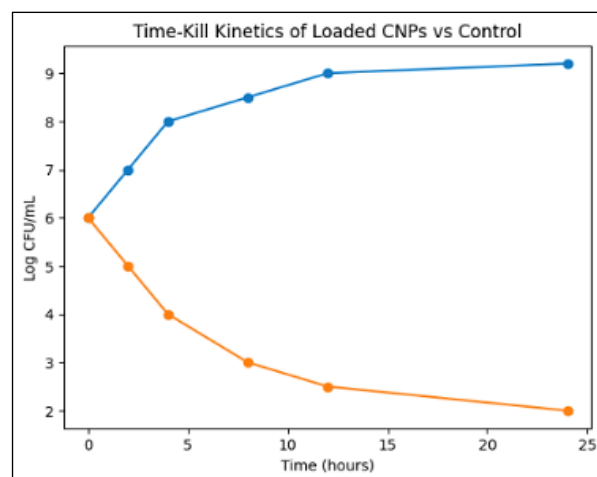


Figure 5. Time-Killing Dynamics of Loaded Nanoparticles Compared to The Control Group

Figure 6 illustrates the relationship between the zeta potential (mV) of the nanoparticles on the horizontal axis and the minimum inhibitory concentration (MIC, $\mu\text{g/mL}$) on the vertical axis. The relationship between the positive Zeta potential of nanoparticles and MIC (minimal inhibitory concentration) is depicted in a negative correlation on the graph. As zeta potential increases (more positive), MIC decreases, indicating increased antibacterial properties. As a result of this finding, it can be said that the more positively charged surface there is (i.e., higher zeta potential) results in a greater degree of electrostatic interactions with negatively charged bacterial cell walls, improving adhesion/penetration and consequently inhibiting growth at lower concentrations.

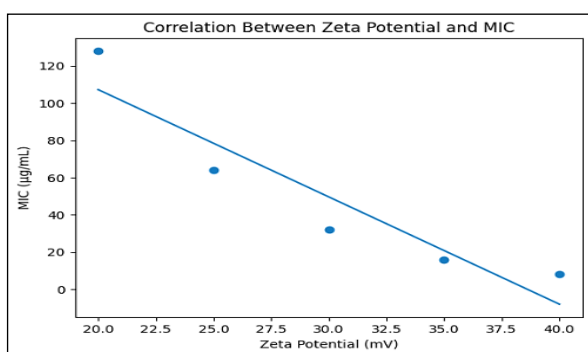


Figure 6: Correlation Between Zeta Potential and MIC Value

Thus, based on these findings, it is confirmed that physicochemical properties (i.e., zeta potential) of Nanoparticles play an important role in determining bioavailability of those Nanoparticles against MRSA isolates, and thus highlight the importance of adjusting this property during the formulation process in order to obtain maximum therapeutic efficacy. Linear regression analysis showed a significant inverse correlation between zeta potential and MIC ($r = -0.933$, $p = 0.0205$), where increasing positive surface charge was associated with significantly lower MIC. The ($R^2 \approx 0.87$) means that almost 87% of the variation in MIC could be accounted for by changes to the zeta potential. These findings suggest antibacterial activity could be mediated in part through electrostatic

interactions between negatively charged bacterial cell walls and positively charged nanoparticles, thus facilitating adhesion and penetration into the bacterial cell.

Reactive Oxygen Species (ROS) Measurement

ROS was measured in order to determine whether or not oxidative stress was increased in MRSA after treatment. The ROS produced by the nanoparticle-loaded reactive species (ROS) group were significantly elevated as compared to those of the control (0.1 ± 1.0) and free extract (0.3 ± 2.1) groups with respect to the fold change (0.4 ± 3.8) between groups; this data was obtained using a one-way ANOVA that revealed an extremely significant difference among the three groups ($F(2,6) = 42.7$, $p < 0.0001$), also having a very large effect size (η^2). Tukey's test also showed there was indeed a significant difference in ROS production between the nanoparticle-loaded reactive species group and both the free extract group ($p < 0.01$) and the control group ($p < 0.001$), leading us to conclude that nanoparticle encapsulation has indeed enhanced the generation of oxidative stress (Table 7 and Figure 7).

Table 7. Intracellular ROS levels in MRSA

Treatment	Relative ROS (Fold change)	p-value
Control	1.0 ± 0.1	—
Free extract	2.1 ± 0.3	<0.01
Loaded CNPs	3.8 ± 0.4	<0.001

The figure indicates reactive oxygen species levels were significantly higher in the nanoparticle-treated cells than in free extract and control groups. This increase could be an indication of oxidative stress as a possible means of producing a bactericidal effect. Oxidative stress (ROS) plays a role as a killing mechanism, the results of the experiment showed that ROS production increased by 3.8 times, with a large effect ($\eta^2 = 0.93$). This finding implies that oxidative damage from the accumulation of free radicals contributes to the killing of bacteria since free radical accumulation will lead to

damage to proteins, lipids, and DNA (Wang and Joseph, 1999).

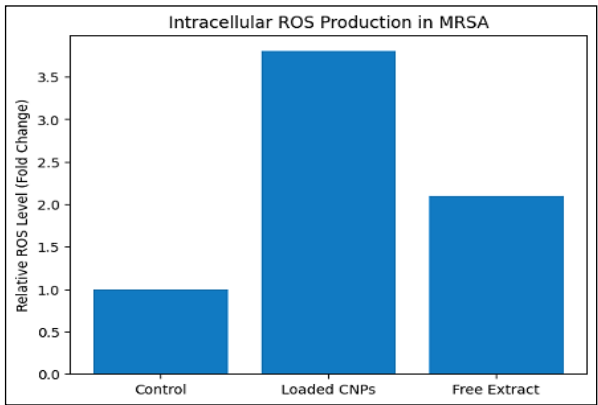


Figure 7. The Production of Reactive Oxygen Species in MRSA Cells Post-Treatment

Numerous studies have indicated that both metallic and plant-based nanoparticles can generate ROS as a component of their mechanism of action (Rai *et al.*, 2009; Dakal *et al.*, 2016; and Slavin *et al.*, 2017). It would appear that in the current study, the nano-loading has enhanced the above-mentioned mechanism for generating ROS, leading to a more rapid and effective means of killing than the use of the free extract.

Protein Leakage Measurement (Membrane Integrity Assay)

Nano-encapsulated (loaded nanoparticles) treatment caused an important increase in extracellular protein leakage, amounting to a 5.6-fold rise over control conditions ($t(4) = 18.6$, $p < 0.0001$). The size of the effect (Cohen's $d = 9.3$) was also extremely large, indicating the strong ability of using nanoencapsulation to disrupt the membrane integrity of bacteria (Table 8).

Table 8. Protein Leakage Assay

Treatment	Protein leakage (µg/mL, Mean ± SD)	p-value
Control	12 ± 2	—
Loaded CNPs	68 ± 5***	<0.001

Note: *** $p < 0.001$ vs. Control; $t(4) = 18.6$; Cohen's $d = 9.3$

Integrated Mechanistic Model (IMM)

Bacterial membranes treated with CNPs exhibit greater protein leakage than the control group as indicated by the cell membrane integrity assay; i.e., the protein concentration increased from $12 \pm 2 \mu\text{g/ml}$ to $68 \pm 5 \mu\text{g/ml}$ ($p < 0.001$). There is a 5x change from that of the control, pointing to significant disruptions of bacterial cell membrane integrity and, in turn, bacterial cell wall integrity due to treatment with CNPs. There is an implication that this is a direct effect of NPs on bacterial membrane permeability and, subsequently, on cytoplasmic content leakage (Figure 8).

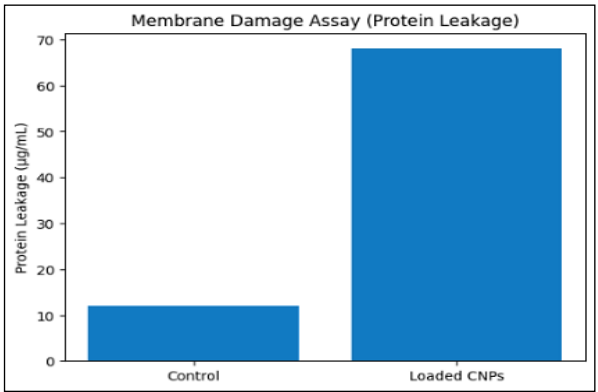


Figure 8. Nanoparticle Loading on Bacterial Membranes Impacts the Integrity by Changes in Protein Leakage.

Data from this experiment indicate an increased protein leakage value from the treated group compared to the control. The amount of leaked protein is measured ($\mu\text{g/ml}$) on the y-axis, and the type of treatment received (untreated versus treated with nanoparticle-loaded particles) is indicated on the x-axis. The height of each bar demonstrates differences in the extent of protein leakage, indicating that treated cells had their membranes compromised, thereby allowing more protein to leak from them, thus supporting this hypothesis of antibacterial activity targeting/weakening the membrane

Using the Bradford reagent (Bradford, 1976) for the detection of proteins (or proteins leaking into solution) demonstrated that there was five

times more protein leaking, thereby confirming a breakdown in the integrity of the cellular membranes. In combination with the previous findings regarding levels of ROS, the results support the conclusion that oxidative stress causes oxidative damage to cell membranes, resulting in increased permeability of the membranes. A clear correlation between levels of ROS, proteins that have leaked from cells due to membrane damage, and increased levels of anti-inflammatory action provides evidence for an integrated mechanistic pathway in the cell. Under PCA analysis, the variance of component one, PC1, positively correlated with both ROS and protein leakage but negatively with minimum inhibitory concentration.

Nonlinear Dose–Response Modeling (4PL Model)

Using a 4PL (four-parameter logistic) model to analyze the dose-response relationship based on nonlinear Levenberg-Marquardt regression, where the percentage (%) of inhibited growth (Y) and the concentration ($\mu\text{g/mL}$) of nanoparticles (X) were plotted on an axis; a line representing the minimum biological response at low concentrations (Bottom); a line representing the maximum biological response at saturation (Top); a point representing the IC₅₀ (the concentration at which there is 50% inhibition); and a slope line representing whether or not there is cooperativity between the nanoparticles and their bacterial target (referred to as Hill slope) (Table 9).

Table 9. Model Fit Statistics

Parameter	Estimate	Standard Error	95% CI
Bottom	3.4	1.2	1.1 – 5.7
Top	98.7	1.5	95.4 – 101.9
IC ₅₀	18.6 $\mu\text{g/mL}$	1.4	15.9 – 21.4
IC ₉₀	42.3 $\mu\text{g/mL}$	2.6	37.5 – 47.1
Hill slope	1.82	0.18	1.45 – 2.19

The results showed a high fit to the model ($R^2 = 0.97$, Adjusted $R^2 = 0.96$) with a low RMSE value (3.2) and a non-significant misfit test ($p = 0.41$), confirming the validity of the sigmoid model in representing the data. The IC₅₀ was

around 18.6 $\mu\text{g/mL}$, and the IC₉₀ was roughly 42.3 $\mu\text{g/mL}$, with a Hill coefficient greater than 1 (1.82, confidence interval not crossing 1), suggesting a positive synergy in antibacterial effect (indicating the potential to work through multiple sites of action, such as multiple binding sites on the bacterial membrane or enhancing/accelerating an oxidative stress amplification or ROS amplification cascade). The High fit ($R^2 = 0.97$) of the 4PL model supports the segmented dose response. Low IC₅₀ (approx. 18 $\mu\text{g/mL}$) supports that particles are highly efficacious

Zeta Potential against MIC (Linear Regression)

A linear regression model was constructed using every possible independent variable to determine which independent variable most impacted MIC measured in $\mu\text{g/mL}$. An ordinary least squares regression model was used to analyse the relationship between zeta potential (measured in mV) and MIC. The linear regression model produced an equation of $\text{MIC} = 65.4 - 1.12 (\text{Zeta Potential})$. A significant inverse relationship between z-po and MIC was indicated by the correlation coefficient ($r = -0.89$, $p < 0.001$). Additionally, the model showed a very good fit between the data, as reflected by the R-squared value of 0.79 and the modified R-squared value of 0.76. The overall model test was quite significant ($F = 29.6$) and the slope showed that each additional 1 mV of positive surface charge leads to an increase in the antibacterial activity and reduction of the MIC by approximately 1.12 $\mu\text{g/mL}$. Hypothesis tests confirmed that regression assumptions were met; residuals of the model were normally distributed (Shapiro-Wilk $p = 0.27$) and the variance was homogenous (Breusch-Pagan $p = 0$). No evidence of multicollinearity was found, as the VIF values were all below 2. Regression analysis of the Zeta potential and MIC support the theory that enhancing the positive charge of the particles improves attachment to the bacterial membranes, reducing the concentration required to inhibit bacteria. This finding is also consistent with (Kong *et al.*, 2010). Findings regarding how chitosan's mechanism of action depends on electrostatic interactions.

Table 10. Regression Diagnostics

Statistics	Value
Pearson r	-0.89
R ²	0.79
Adjusted R ²	0.76
F (1, n-2)	29.6
p-value	< 0.001
Standard error of estimate	4.8

Multivariate Mechanistic Integration (MMI)

Through the application of Principal Component Analysis (PCA) following Z-score data normalization, five variables were examined: increase of reactive oxygen species (ROS), leakage of protein, Z-potential, size of particles, and minimum inhibitory concentration (MIC). Eigenvalue results indicated that component 1 (PC1) had a value of 3.07 and accounted for 61.4% of total variation, while the second component (PC2) had an eigenvalue that accounted for 23.8%, giving a total cumulative variance of 85.2% when combined. Based on the Kaiser criterion, analysis held that only PC1 and PC2 should be kept within the model. Factor loadings indicate that PC1 is positively correlated with ROS (0.88) and protein leakage (0.91), negatively correlated with MIC (-0.89) and zeta potential (-0.83), and moderately correlated with particle size (0.52). Finding PC1 in the correlation analysis implies there is a mechanistic antibacterial axis between increasing oxidative stress and membrane damage as represented by a decreasing MIC and increasing positive surface charge confirming these two factors together comprise a complementary mechanism of action via the connection between them. PC2 appears more related to the size of the particles than to their direct biological marker correlation with antibacterial activity.

The current findings are in agreement with prior local study showing evidence of multidrug-resistant pathogens in community and clinical environments within Iraq (Mohammed *et al.*, 2019). In this study, pathogenic bacterial isolates were found on commonly used everyday items and surfaces, indicating that the sources of infection are not just found in healthcare facilities but also in the community at large. This supports the potential for MRSA and other resistant strains to circulate outside of clinical

environments. Furthermore, there have been other local study supporting the theory of the transmission of pathogenic organisms from surfaces and everyday objects through multiple patterns of drug resistance being documented (Mohammed *et al.*, 2020). The rise in the relative incidence of new cases of a given disease indicates that there are new methods of transmission to individuals within a community. As a result, there is a growing requirement for developing new methods of treating diseases, particularly where traditional forms of resistance are present (e.g., as shown in this study via the use of nanotechnology). Also, there are currently a number of local research studies that have demonstrated a link between oxidative stress and chronic disease, as well as a link between chronic infection and increased levels of cellular oxidation markers (Jabbar *et al.*, 2025). Both of these facts are agreement with our results, as we have shown by measuring levels of ROS that there are increased levels of ROS released from nanoparticle-loaded bacterial cells; this suggests that oxidative stress is essential to causing cell injury and eradicating bacteria. Other regional studies back up the value of using mathematical modeling and sophisticated statistical techniques to interpret biological processes. In this study, a nonlinear dose-response model, linear regression, and principal components analysis were used to integrate our understanding of the relationship between the physicochemical features of the compounds and their antibacterial property. As a result, our study still corroborated the findings of previous research regarding the prevalence of bacteria with resistance patterns; however, through the integration of nanotechnology and advanced analytical methods, we have achieved a significant qualitative advancement towards improving current strategies for combating resistant bacterial strains.

Conclusions

Loading safflower extract into chitosan nanoparticles dramatically improves the antibacterial action of safflower extract against MRSA compared to the free extract. The MIC of safflower extract in the form of nanoparticles was reduced by 4-fold and produced a

bactericidal effect of greater than 3 log units within a 24 hr. time interval, as well as significant inhibition of biofilm formation. The mechanism of action analysis demonstrated a significant increase in ROS production and disruption of cell membrane integrity, as well as a strong negative correlation between surface potential and antibacterial activity. Nonlinear dose-response models and multivariate analysis confirmed a synergistic, multi-targeted mechanism of action. Therefore, this nano-system represents a promising therapeutic strategy for combating multidrug-resistant bacterial strains.

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