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RESEARCH ARTICLE

Evaluating the Antibacterial and Anti-Biofilm Properties of Chitosan Nanoemulsion Containing *Euphorbia hebecarpa* Plant Extract Against *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

The use of nanoparticles as drug-carrying systems in medicine has received much attention, and on the other hand, the use of plants with medicinal properties has advantages over synthetic drugs. This study aims to improve the antibacterial and anti-biofilm properties of *Euphorbia hebecarpa* plant extract using chitosan nanoparticles. Extraction were performed using the modified maceration and methanol solvent. The agar disc diffusion method was carried out to investigate the antimicrobial effects and MIC at an optical density (OD) of 630 nm. The anti-biofilm agent's influence was measured at OD 570 nm, and the metabolic activity of bacteria in the damaged biofilm was determined using triphenyl tetrazolium chloride (TTC) staining. The nanoparticles were obtained by following the ionic gelation method and its characteristics were evaluated. Chitosan nanoparticles loaded with plant extract were found to be spherical shape with hydrodynamic diameter of 142 nm, a polydispersity index (PDI) of 0.184, a zeta potential of +28.6 mV. It was determined that the loading capacity (% LC) and encapsulation efficiency (% EE) percentages were 33% and 40%, respectively. The diameter of the inhibition zone in the agar disc diffusion test for both bacteria using nanoparticles loaded with the extract increased compared with control groups. Also, at a concentration of 4 mg/mL of loaded nanoparticles, 49.33% of *Escherichia coli* biofilm and 62.7% of *Staphylococcus aureus* biofilm were destroyed. The data obtained from the tests performed in this study show that the use of *Euphorbia* plant extract in the form of chitosan nanoparticles can have more antimicrobial and anti-biofilm effects.

1 | Introduction

Nanoparticles, with their high surface-to-volume ratio, small size, and surface charge, have unique physicochemical features and have found wide applications in biology. So that the number of articles published in this field has been growing over the years, which can indicate the importance of examining nanoparticles [1]. Nanoparticles can be produced from a variety of compounds, including chitosan.

Chitosan is a linear polysaccharide polymer that has been investigated for its antibacterial, antifungal, and antioxidant qualities as well as its biodegradability and biocompatibility [2, 3]. Explaining chitosan's antibacterial effect, it can be noted that

this polymer is unable to pass through the cell membrane due to its high molecular weight, and by building a layer on this membrane, it limits the entrance of bacteria-required chemicals into the cell. As a result, this process damages the bacterial cell [2].

Current methods for the treatment of microbial biofilms are mostly based on the use of antimicrobial compounds of chemical origin. Although these compounds can effectively remove microbial biofilms, their most important problem is that they have very high side effects, and on the other hand, antimicrobial resistance has been created against them in recent years, which will reduce their effectiveness in the future.

The use of nano-compounds for the treatment of microbial biofilms has been emphasized in recent years. For example, silver nanoparticles have been used, but nanoparticles of chemical origin also have side effects on humans. For this reason, the desire to use natural and herbal compounds that have less side effects and more effectiveness has increased. But a major problem that plant compounds have is their low stability in the external environment, but if they are in the form of nanoparticles and are accompanied by a carrier such as chitosan, their durability increases significantly. Another advantage of using plant nano extracts with chitosan carrier is that the antimicrobial resistance against them is low.

Microbial biofilms, bacterial communities embedded in an extracellular polymeric substance matrix that is formed during extreme environmental conditions. These structures are resistant to antibiotic treatment and the host's immune response, which can lead to chronic infections [4, 5]. Chronic infections caused by *Staphylococcus aureus* and *Escherichia coli* are chronic rhinosinusitis and urinary tract infections, respectively [5].

Because plants lack the immunity system, they produce some chemical compounds to protect themselves against environmental pathogens [6]. *Euphorbia* is a plant that often grows in tropical and subtropical regions [7]. Research has shown that the bioactive compounds identified from this plant have an enzyme inhibitory effect as well as damage to DNA [8]. There is a report on the antimicrobial effect of *Euphorbia* plant extract on *E. coli* and *S. aureus* bacteria, the cause of which is the presence of tannins, alkaloids, and flavonoids in plant extracts [9].

The aim of this investigation was to improve the antibacterial and anti-biofilm properties of *Euphorbia* plant extract-chitosan nanoemulsion against two human pathogens: *S. aureus* and *E. coli*.

2 | Materials and Methods

2.1 | Extraction

Euphorbia hebecarpa plant was collected from the area around the Assir region in the south of Saudi Arabia. Extraction was performed by using modified maceration with methanol solvent. In this method dried and powdered plant in methanol solvent 96% with a ratio of 1:10 v/w were used. After 24 h at 40°C for the removal of large parts of the plant No. 1 Whatman filter paper was used. After removing the solvent, the extract powder was collected and stored [10].

2.2 | Microorganisms

The Gram-negative bacteria *E. coli* ATCC 35218 and the Gram-positive *S. aureus* ATCC 1189 were selected for the study.

2.3 | Preparation of Nanoemulsion

The ionic gelation process was employed to create chitosan nanoparticles loaded with plant extract. Calvo et al. [11] developed the ionic gelation process, a chemical method for producing microparticles, or NPs, which relies on electrostatic interactions between ions with different charges. Typically, 30 mg chitosan (an average molecular weight of 80%–85%) was dissolved in 0.5% (v/v) acetic acid for 30 min. Chitosan solution using filters Millipore 45 μm was filtered to remove chitosan unresolved. Two-hundred milliliters of Tween 80 as surfactants to enhance the stability of the nanoparticles and to obtain a homogeneous solution was used. To make the nanoemulsion, 10 mg of diluted herbal extract in 500 μL of ethanol was added to the chitosan solution and stirred for 30 min. Afterward, 100 mL of aqueous tripolyphosphate (TPP) was gradually added. The NPs morphological were judged by scanning electron microscopy (SEM) (JEOL) and particle size was measured through dynamic light scattering (DLS) using the Zeta sizer HSA 3000 (JEOL) at 630 nm, scattering angle 90° and 25°C.

2.4 | Determination of Herbal Extract Loading Into Chitosan Nanoparticles

Encapsulation efficiency (EE) and loading capacity (LC) percentages were estimated using Equations (1) and (2).

$$\mathrm{EE}\,(\%) = \frac{C0 - C1}{C0} \times 100 \tag{1}$$

Equation (1): EE determination.

$$\mathrm{CL}\,(100\,\%) = \frac{W\ (\text{loaded extract})}{W\ (\text{Total nanoparticles loaded in chitosan})} \times 100 \tag{2}$$

Equation (2): LC determination.

2.5 | Antimicrobial and Anti-Biofilm Assay

The Bauer-Kirby disc diffusion method [12] was utilized to assess the antibacterial capabilities of the nanoparticles. Test bacterial stock cultures were grown in NB medium for 18 h at 37°C. The final cell concentration was set at 10^8 colony-forming units (CFU)/mL using the McFarland turbidometer as a reference [13]. The suspension was standardized by adjusting the optical density to 0.13 at 600 nm (VarianCary50, America). About 500 μL of the bacterial solution was added to each Petri dish containing Mueller Hinton Agar and then spread throughout the agar. Incubation was performed for 18 h at 37°C.

The micro-broth dilution method, which is recommended by the Clinical and Laboratory Standards Institute [12], was used to determine the MICs and MBCs using NB as the test medium. Overnight, bacterial cultures were diluted to yield a final

concentration of 5×10^5 CFU/mL. After that, corresponding volumes of varied extract concentrations were added to tubes along with samples made from sequential two-fold dilutions ranging from 0.05 to 50 mg/mL. To make these solutions, 100 mg/mL of extract stock concentration was diluted in sterile culture medium (NB). The MIC was determined after 18-h incubation at 37°C using the lowest concentration of substance that stopped visible growth. Bacteria in NB and bacteria in NB containing ciprofloxacin serves as negative and positive control, respectively. The MBC was calculated by spreading 150 μL on an MHA plate from a sample with no apparent growth and incubating it for 18 h at 37°C.

Anti-biofilm effect of nanoparticles using a microtiter plate and the plate of 96 wells was performed. Microbial biofilms of both *E. coli* and *S. aureus* bacteria formed after 48 h at 30°C, and then the wells were treated with extract-loaded nanoparticles, non-extracted chitosan nanoparticles, and plant extracts for 1 h. For staining of the biofilm from 250 μL of crystal violet 2% was used for 5 min and OD was obtained at 570 nm. Equation (3) calculates the reduction of the biofilm [13].

$$\text{Reduction Percent} = \left[\frac{(C-B)-(T-B)}{(C-B)}\right] \times 100 \quad (3)$$

Equation (3): Reduction percent. *C*: Average OD of untreated microbial biofilm wells, *B*: Average OD of broth cultures wells, *T*: Average OD of treated wells.

The metabolic activity of bacteria in biofilm was investigated using TTC labeling. Pre-formed biofilms were rinsed twice with PBS before adding extracts (12.5–50 mg/mL) and incubated for further 24 h at 37°C. After adding 50 μL of TTC solution to each well, the reaction was allowed to occur in the dark at 37°C for 3 h. A microtiter plate reader set to 490 nm was used to determine the final absorbance value. The percentages of reduced biofilm metabolic activity in the presence of varied extract concentrations were calculated using the formula described above.

2.6 | Statistically Analysis

The data pertaining to parameter differences between the treatment and control groups were analyzed using the Duncan multiple range test. The statistically significant *p* values for each test conducted in triplicate were determined as *p* values less than 0.05, 0.01, and 0.001. *p* values less than 0.05 were considered statistically significant.

3 | Results and Discussion

3.1 | Plant Identification

The collected plant samples were transported to Herbarium plant identification was done by plant taxonomist Dr. Mirtajadini. The voucher specimen number is HSBUK3799 (Herbarium of King Saud University). This plant also stores in this herbarium.

3.2 | Preparation of Nanoparticles

Due to the nature of polysaccharides in high temperatures, chitosan polymer nanoparticles have lost their stability and therefore micro-particles with very low production efficiency have formed and adhered to each other. Examining the ratio of chitosan to TPP and not using a surfactant, the concentration of 16 mg/mL TPP and the ratio of 3–12 chitosan to TPP had the best results in the formation of nanoparticles. It should also be noted that due to the non-use of surfactant, the stability of nanoparticles has been less than 1 h. The best pH was obtained for the synthesis of nanoparticles for chitosan 3.6 and TPP 9.3, and experiments showed that changing pH of chitosan and TPP led to instability and particle accumulation.

The use of a 0.5% (v/v) concentration of acetic acid led to a better milky suspension. Also, with the increase in the concentration of Tween 80 to 4% (v/v) of the nanoparticles, the stability of the nanoparticles increases, and after 72 h, no accumulation of nanoparticles is observed in the suspension.

3.3 | Physicochemical Characterization of Nanoparticles

SEM imaging showed an almost spherical homogeneous structure for extracted chitosan nanoparticles and non-extracted nanoparticles, the results of which are display in Figure 1.

The results of DLS were determined according to what is shown in Figure 2 for chitosan nanoparticles and nanoparticles loaded with extract in terms of the average size of 138 and 142 nm, as well as for polydispersity index (PDI) 0.207 and 0.184, respectively. Besides, the dispersion is uniform for both groups and the increase in the size of nanoparticles loaded with the extract compared with chitosan nanoparticles alone can be due to the use of the extract in the formulation of nanoparticle synthesis.

The zeta potential of chitosan-loaded nanoparticles equal to +28.6 mV in Figure 3 shows that this positive potential causes the nanoparticles to stabilize over time and can also prevent the nanoparticles from connecting and accumulating with each other. Also, the FTIR image of the nanoparticles were shown in Figure 4.

The amount of chitosan loaded nanoparticles was calculated to be 8 mg. Therefore, the synthesis efficiency of nanoparticles is 27%. The efficiency of encapsulating (% EE) or trapping efficiency of plant extracts 40% and loading capacity (% LC) was 33%. So, 4 mg of extract of *Euphorbia* plant in chitosan nanoparticles was trapped. The reason why the EE is not complete and is only 40% is due to the fact that too much of the free extract is not absorbed by the chitosan nanoparticle and is removed in the environment. Most researchers have reached the same efficiency.

3.4 | Antimicrobial and Anti-Biofilm

The information obtained to determine the antimicrobial effect was reported in Table 1 and Figure 5. Based on this,

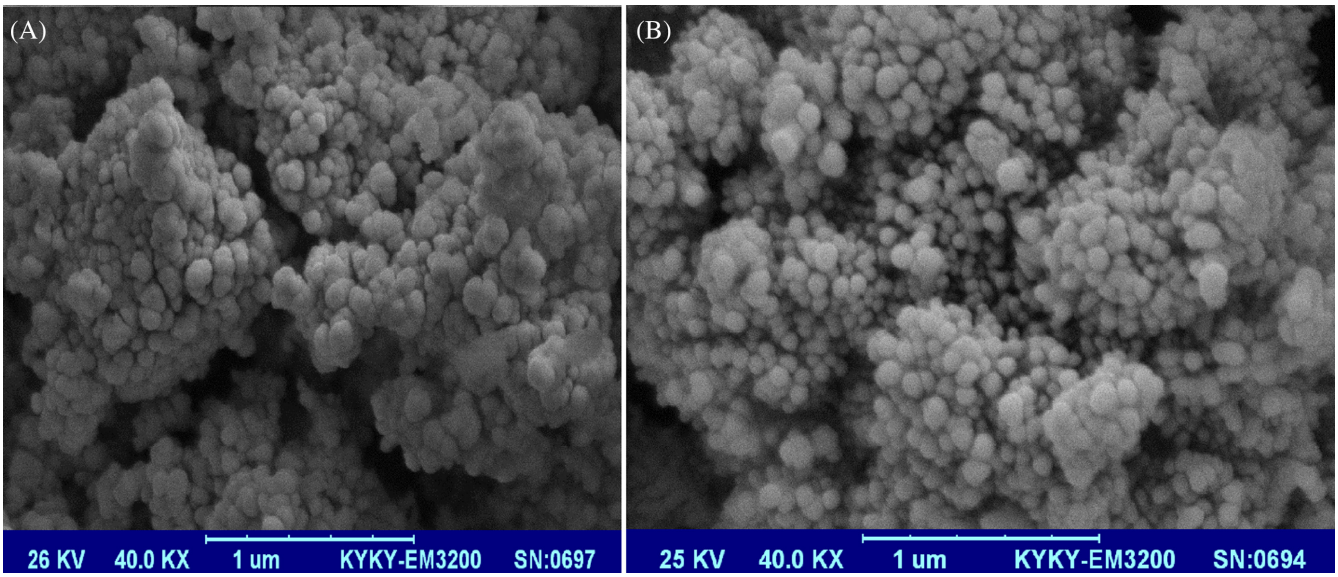

FIGURE 1 | SEM analysis results. (A) Chitosan nanoparticles not loaded. (B) Chitosan nanoparticles loaded with *E. hebecarpa* extract.

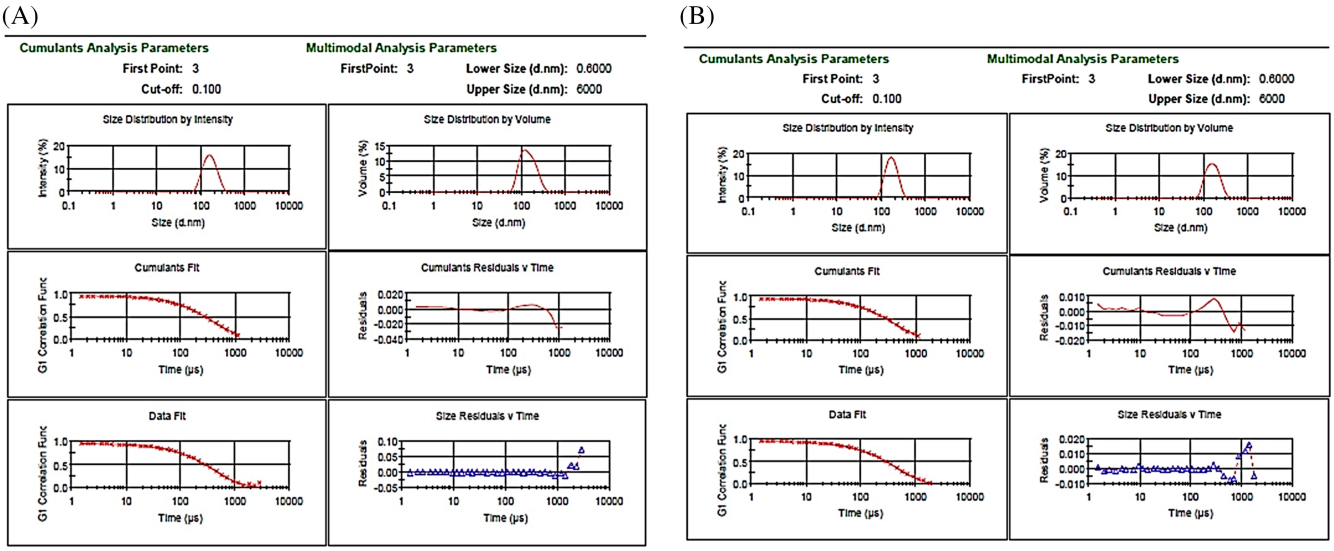

FIGURE 2 | Results related to DLS study. (A) Dispersion size of chitosan nanoparticles without extract and (B) with plant extract.

in the agar disc diffusion test, the effect of loaded nanoparticles with extract was higher than unloaded nanoparticles and plant extract on both bacteria and it was also found that this effect was greater on *E. coli* bacteria than *S. aureus*. The relationship between the concentration of each sample and inhibit the growth of bacteria was drawn graph in Figure 5. The only MIC detected for nanoparticles loaded with the extract was against the *E. coli* at a concentration of 8 mg/mL. In other groups and concentrations, MIC was above 10 mg/mL and unrecognizable.

The results of the anti-biofilm effect of the samples were classified and reported in Table 2. Three concentrations of 1, 2, and 4 mg/mL were used, and after treating the biofilms with each group, the number of bacteria removed was determined. With a little care, the amount of live bacteria can be detected, which can multiply even after biofilm destruction. Also, the statistically analysis of the results were shown in Table 3.

Herbal bioactive compounds have been considered in the discovery and development of new drugs due to their easy access and variety of chemical compounds [14]. Furthermore, using nanoparticles as a drug delivery technique has several advantages, including improved pharmacokinetic and pharmacodynamic properties [15]. Therefore, it can be said that the use of plant compounds in the form of nanoparticles can lead to the strengthening of the desired effect.

The antimicrobial effect of chitosan polymer against Gram-negative bacteria such as *E. coli* has been attributed to the dissolution of low molecular weight chitosan in water, as well as the positive charge of the polymer, which creates an electrostatic interaction with the anionic surface of *E. coli* bacteria. The interference created can disrupt the cell membrane. In this regard, the antimicrobial effect against Gram-positive bacteria such as *S. aureus* due to polymer binding is attributed to biomolecules such as DNA and RNA [2]. This could be an

System

Temperature (°C):	24.9	Zeta Runs:	10
Count Rate (kcps):	406.7	Measurement Position (mm):	2.00
Cell Description:	Clear disposable zeta cell	Attenuator:	9

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	28.6	Peak 1:	28.6	100.0	5.12
Zeta Deviation (mV):	5.12	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm):	0.719	Peak 3:	0.00	0.0	0.00

Result quality : Good

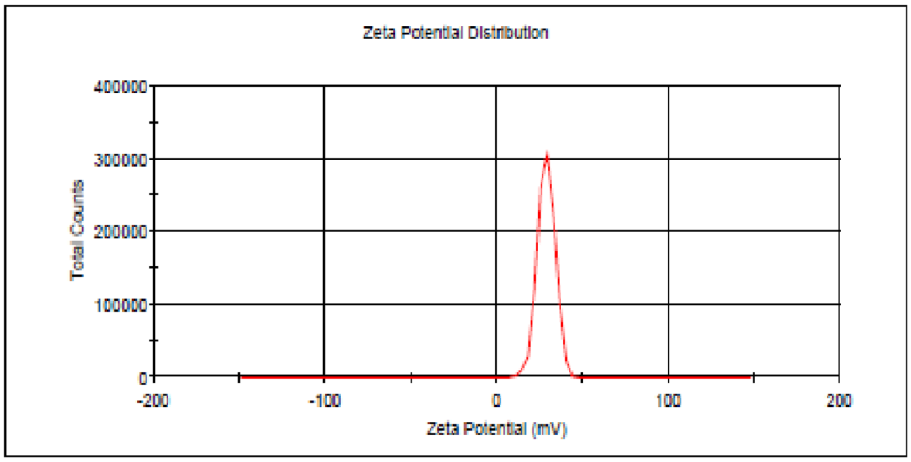

FIGURE 3 | Zeta potential of chitosan nanoparticles loaded with extract (+28.6 mV).

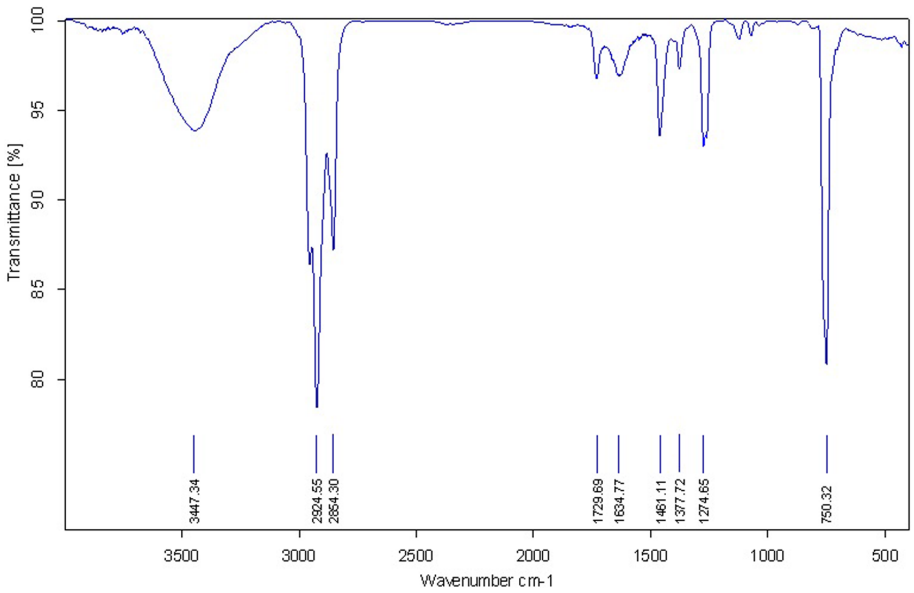

FIGURE 4 | The FTIR image of nanoemulsion.

explanation for the antimicrobial effect of chitosan nanoparticles without loading with the extract according to what is shown in Figure 4, and also indicate the selection of the appropriate polymer for this study. Although the results of both the agar disc diffusion and the MIC test were better for *E. coli* than *S. aureus*, in the study of anti-biofilm activity, it can be stated that the effect on the *S. aureus* was more obvious. So that at concentrations of 4 mg/mL loaded nanoparticles, the biofilm degradation of *E. coli* and *S. aureus* was 49% and 62%, respectively.

In a similar study, Madureira et al. [16] studied chitosan nanoparticles loaded with phenolic compounds with a size of 300–600 nm and zeta potential of 20–30 mV to investigate the antimicrobial

effect on some food pathogenic bacteria. According to their results, all bacteria showed an inhibition percentage above 60%–90%, which of course had the highest effect on *E. coli* and *Bacillus cereus* bacteria and the lowest on *Salmonella typhimurium*. Sathiyabama et al. [17] synthesized chitosan nanoparticles from tea extracts and investigated their antibacterial efficacy against certain pathogenic microorganisms. Their research suggests that biosynthesized CTNp can be employed to manage the majority of infections and germs. Divya et al. [18] by preparing chitosan nanoparticles by ionic gelation method, studied some pathogenic bacteria, including *E. coli* and *S. aureus*. According to their report, the nanoparticles produced compared with the control groups have antimicrobial and anti-biofilm effects against all the tested bacteria, which is due to the presence of primary amine groups in chitosan are mentioned. Wu et al. evaluate the antibacterial and antibiofilm activity of the favored fractions and compounds of *E. humifusa* against *S. aureus*. Their findings indicated that the extract from this plant destroys cell integrity, increases membrane permeability, and inhibits biofilm-related gene expression. According to an analogous research by Du et al. intending to study the antimicrobial effect of chitosan nanoparticle loaded copper against *E. coli* bacteria, their results showed that the amount of MIC and MBC decreased by 21 and 42 times, which is due to damage to the bacterial cell membrane by chitosan nanoparticle loaded copper [18].

TABLE 1 | The results of the agar disk diffusion (mm).

	E. coli	***S. aureus***
E. hebecarpa plant extract (10 mg/mL)	8 ± 0.8	9 ± 0.3
Chitosan nanoparticles (10 mg/mL)	8 ± 0.6	7 ± 0.8
Chitosan nanoparticles loaded (10 mg/mL)	13 ± 0.9	12 ± 0.3

In this study, the ratio of 3–1 chitosan to TPP was observed according to the findings of DLS and SEM of the best size, diameter, zeta potential, and morphology of nanoparticles. Fan et al. employed TPP as a low molecular weight binder to investigate the ionic gelation method for producing chitosan nanoparticles. As a result, nanoparticles with 138 nm hydrodynamic diameter, 0.026 PDI, and a zeta potential of +35 mV were produced. The

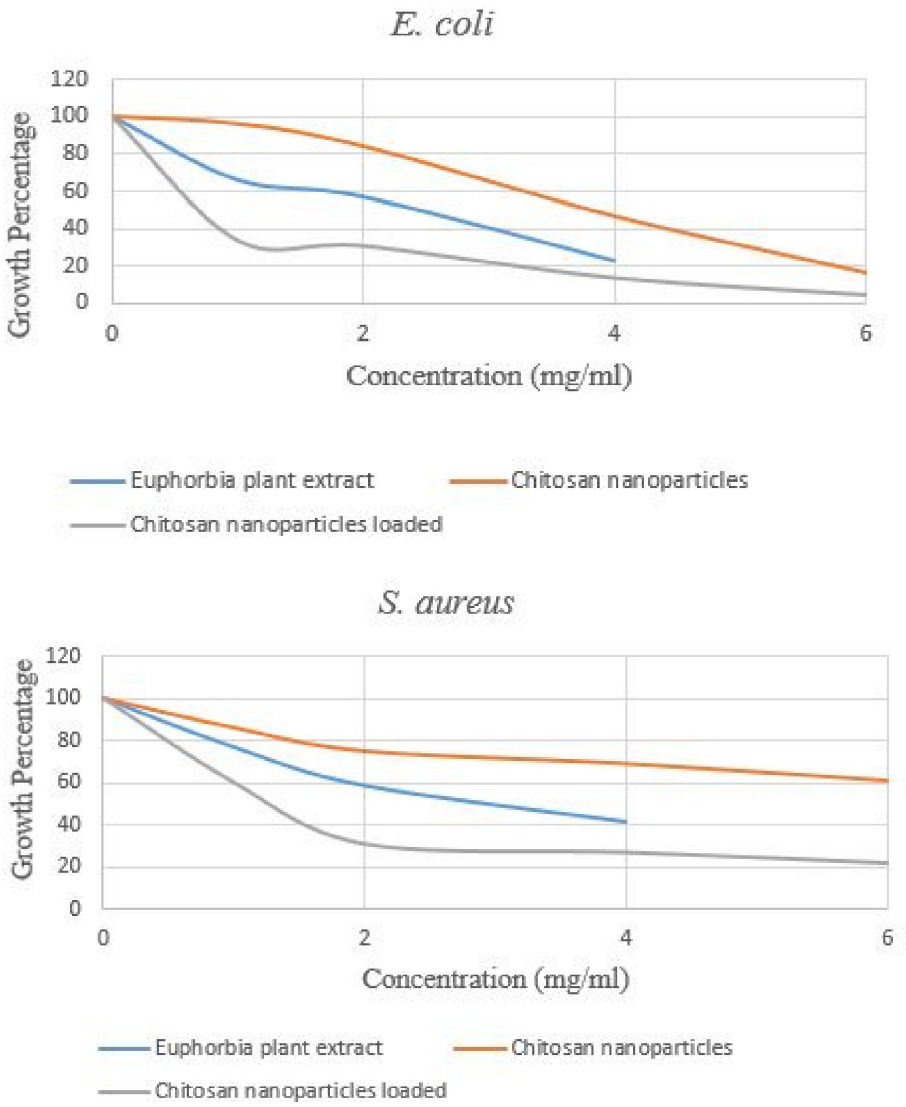

FIGURE 5 | The results of the MIC. The chart shows a decrease in the growth rate due to an increase in concentration in all three groups.

TABLE 2 | The results of the anti-biofilm effect.

		E. hebecarpa plant extract			Chitosan nanoparticles			Chitosan nanoparticles loaded		
		1 (mg/mL)	2 (mg/mL)	4 (mg/mL)	1 (mg/mL)	2 (mg/mL)	4 (mg/mL)	1 (mg/mL)	2 (mg/mL)	4 (mg/mL)
E. coli	Biofilm destruction	13.5	25.53	40.14	4.04	26	36.12	21	37.75	49.33
	Bacterial removal rate[a]	17	26.60	49.08	21.10	36.08	45.38	52.93	54.12	61.2
S. aureus	Biofilm destruction	26	36.54	55.34	34	38	42.48	51.72	58.6	62.7
	Bacterial removal rate[a]	44	50.12	69.75	42.96	52.13	55	58	59.41	64.31

[a]The rate of elimination of released bacteria after biofilm treatment.

TABLE 3 | Data from various experiments were statistically analyzed using Duncan's test.

	df			MS			Sig		
Sources change	**Biofilm formation**	**Destruction biofilm**	**Inhibition enzyme**	**Biofilm formation**	**Destruction biofilm**	**Inhibition enzyme**	**Biofilm formation**	**Destruction biofilm**	**Inhibition enzyme**
1. Bacteria type	5	5	5	0.123	0.047	0.048	*	**	**
2. *E. hebecarpa* plant extract	1	1	1	0.091	0.247	0.002	—	—	—
3. Chitosan nanoparticles	5	5	5	0.0121	0.090	0.037	*	***	*
4. Chitosan nanoparticles loaded	2	2	3	0.014	0.065	0.003	*	**	—
Total	25	25	32						

Note: *Significant level at 0.01; **significant level at 0.1; ***significant level at 0.05.
Abbreviations: df, degree of freedom; MS, min square; Sig, signification.

finding indicated that by lowering the acetic acid concentration and ambient temperature during the cross-linking process, the size of the nanoparticles could be greatly reduced [19]. As discussed in previous reviews, the inclusion of chitosan nanoparticles enhances the antibacterial and anti-biofilm effects. Clinical application of the finding of the current study can be develop some antibacterial agents for treatment of pathogenic bacteria specially for bacteria that formed robust biofilm such as teeth plaque. Also, construction of a solution for destroy the biofilm in medical instrument that used in ICU is another application of the finding of current study. Future research directions of the current research are in vivo studies or exploring the mechanism of action, to advance the translational aspects. Also, study the anti-fungi activity of these nanoparticles can be another field of study of this research.

4 | Conclusion

The data obtained from the tests performed in this study show that the use of *Euphorbia* plant extract in the form of chitosan nanoparticles can have more antimicrobial and anti-biofilm effects.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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