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Curcumin-loaded Chitosan Tripolyphosphate Nanoparticles as a safe, natural and effective antibiotic inhibits the infection of *Staphylococcus aureus* and *Pseudomonas aeruginosa in vivo*

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Background: Curcumin as a yellow natural compound extracted from turmeric root is known it as an antibacterial agent. One of the nanoparticles ability is to decrease the defects of usual drug delivery systems. Chitosan is a low toxic, biodegradable, biocompatible and safe polymer which is used in production of nanoparticles. Nanoparticles like chitosan-tripolyphosphate (TPP) are able to increase antibacterial properties of curcumin.

Materials and Methods: Curcumin-loaded chitosan-TPP nanoparticles containing chitosan, curcumin and TPP salt were synthesized by ionotropic gelation methods. First, the skin of anesthetized mice was inoculated with *staphylococcus aureus* and *pseudomonas aeruginosa* suspension. Then the infected mice were treated with curcumin-loaded chitosan-TPP nanoparticles for 3 days. Following that, antibacterial characteristics of the mice treated with curcumin-loaded chitosan-TPP nanoparticles were evaluated by bacterial culture of these mice.

Results: Our results showed the size of 160 ± 10 nm and the charge of +7 ± 2 mV in curcumin-loaded chitosan-TPP nanoparticles. These nanoparticles were also spiral shape. The encapsulation efficiency of curcumin in chitosan-TPP nanoparticles was 75 ± 2%. Bacterial culture showed that curcumin-loaded chitosan-TPP nanoparticles inhibited *staphylococcus aureus* and *pseudomonas aeruginosa* growth.

Conclusions: Our study demonstrated that curcumin-loaded chitosan-TPP nanoparticles can be utilized as a potent agent in treatment of *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections.

Keywords: Chitosan-TPP; Curcumin; Nanoparticles; *Pseudomonas aeruginosa*; *Staphylococcus aureus*

1. Background

Curcumin as a natural yellow pigment and a nontoxic, bioactive agent of turmeric separated from the root of *Curcumin Longa* has been utilized in traditional medicine. Besides, it has been observed that curcumin as a strong antibacterial drug is authenticated to inhibit the growth of several bacteria including *Staphylococcus aureus* and *Pseudomonas aeruginosa* is well-known as it a good candidate for treating inflammatory diseases (1, 2) The ability of this drug in skin wound healing has been demonstrated on the other hand. In order to enhance wound healing, curcumin can increase biosynthesis of extracellular matrix proteins such as collagen (3, 4).

Chitosan is a linear polysaccharide derived from crustacean shells and fungi cell walls. It has been used as a biocompatible and safe material in drug delivery systems (5-7). It has been recently used in bandages and other haemostatic agents (8, 9). In addition, chitosan due to the ability of preventing the wound from being infected and dehydrated can optimize suitable conditions for healing (10). It has also been utilized as an antimicrobial agent that prevents the spread of infections into surrounding area (11, 12). Many studies have

described the role of chitosan as a wound-healing accelerator. Chitosan could accelerate coagulation and enhance the functions of inflammatory cells (13-16). Moreover, it has been reported that chitosan could increase the tensile strength of wounds (17).

Nanoparticles are made from different biodegradable materials and their dimensions are generally less than 500 nm (18, 19). Chitosan nanoparticles may be more efficient than chitosan solution at enhancing drug activity (20-23). Thus, chitosan-tripolyphosphate (TPP)-nanoparticles have been widely applied to deliver drugs across tissues. Overall, using chitosan-TPP nanoparticles as a nano-system can increase curcumin delivery in infectious tissue.
(18, 24, 25).

2. Objectives

In the present study, we optimized an optimal method for curcumin loading in chitosan-TPP nanoparticles and investigated the antibacterial activity of curcumin-loaded chitosan-TPP nanoparticles on *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections.

3. Materials and Methods

3.1. Animals

Female BALB/c mice were provided from Pasteur Institute of Iran (Iran, Tehran). All experiments were performed on 6 to 8 week-old mice in accordance with the guidelines of the Medical Ethics Committee of Baqiyatallah University of Medical Sciences (BMSU).

3.2. Preparation of Bacterial Suspension

Staphylococcus aureus (ATCC: 25923) and *Pseudomonas aeruginosa* (ATCC: 27853) were grown for 18 h in luria broth (LB) medium. The bacterial cells were finally collected, washed, and resuspended in sterile phosphate-buffered saline (PBS, pH 7.4). They were adjusted to a cell suspension of 10^7 colony forming unit (CFU)/mL using a UV spectrophotometer (Biophotometer, Eppendorf, Germany) in 620 nm wavelength.

3.3. Curcumin-loaded chitosan-TPP nanoparticles synthesis

Preparation of nanoparticles by ionotropic gelation method is based on electrostatic interaction between negatively- and positively-charged molecules such as poly anionic and cationic polymers. In the case of curcumin-loaded chitosan-TPP nanoparticles, the amino groups existed on chitosan interacts with anionic groups of TPP salt. Stock solution of chitosan was made at 1 mg/mL in acidified distilled water (DW) and TPP was made at 1 mg/mL in DW. First, the chitosan stock solution (1 mL) was stirred for 10 min and its volume got adjusted to 1.5 mL with DW. Next, we added 5 μL tween 80 to curcumin stock (1 mg/mL) solved in ethanol. Then, curcumin was added to the chitosan solution. Finally, TPP solution as a cross linker (100 μL) were added to emulsified-curcumin-chitosan solution in a dropwise manner. The obtained solution was stirred for 30 min and centrifuged at 4000 g for 5 min. At last, the supernatant was transferred into a new tube and kept for subsequent analysis.

3.4. Curcumin-loaded chitosan-TPP nanoparticles characterization

The samples were sonicated for 5 min in bath Wisd, WUC-D10H sonicator (Dihan, South Korea) before being analyzed and they were immediately used for measurements. The size and zeta potential of prepared nanoparticles were characterized by photon correlation spectroscopy (PCS) using a Malvern Zetasizer ZS series and Scattering Particle Size Analyzer (Malvern Co, UK). Following that, the shape, size and aggregation phenomena of curcumin-loaded chitosan-TPP nanoparticles were measured by atomic force microscopy (AFM) (NVB-100, Olympus, Japan) and transmission electron microscopy (TEM) (Zeiss EM900, Carl Zeiss AG, Germany). Next, the fourier transforms infrared (FTIR) spectra of curcumin, chitosan and curcumin-loaded chitosan-TPP nanoparticles were assessed using Nicolet IR100 FTIR Spectrometer (Thermo, USA). Ultimately, the samples were mixed with pure potassium bromide (KBr) as the background and compressed into discs using a manual tablet press.

3.5. Evaluation of curcumin-loaded chitosan-TPP nanoparticles encapsulation

To obtain nano-system with a maximum ratio of drug loading, different weight/weight ratios of chitosan/curcumin were tested. Therefore various amounts of curcumin were dissolved in a certain

amount of chitosan-TPP nanoparticles. Subsequently the product was centrifuged at 20000 rpm for 25 min and the supernatant of centrifuged curcumin-loaded chitosan-TPP nanoparticles formulation was checked for absorbance spectra by a spectrophotometer (Amersham Biosciences, Uppsala, Sweden) at 432 nm. The loading efficiency was calculated using the following equation:

Encapsulation efficiency (%) = [(Total amount of curcumin-Nonencapsulated curcumin) / Total amount of curcumin] × 100%

3.6. Determination of curcumin release profile from nanoparticles

The release of curcumin from curcumin-loaded chitosan-TPP nanoparticles was evaluated using phosphate buffer (pH 7.4) and citrate buffer (pH 5.4) at 37°C. 1 mL of the solution was poured into a dialysis bag (Spectrapor, MW cutoff 3500 g/mol) and placed into 100 mL of phosphate buffer (pH 7.4) and citrate buffer (pH 5.4) severally. Afterward, 100 μL tween 80 as an emulsifier agent was added in order to prevent the possible sedimentation of released drug. The release study was carried out at 37ºC applying a shaking water bath (GFL, Burgwedel, Germany) and at the dedicated time intervals of 0, 0.5, 1, 1.5, 2, 4, 8, 12, 24, 48, 72 and 96 hours, the sampling was performed. In each time point, 500 μL of the sample was elicited, and then replaced by 500 μL buffer. Then samples freeze dried and then dissolved in 2 mL of methanol. Finally all samples were examined by making use of spectrophotometer (Amersham Biosciences, Uppsala, Sweden) to determine the quantity of releasing curcumin. The accumulated release was calculated utilizing the following equation:

$$R= [V \sum^{n-i} (C_i + V_0 C)] / m_{drug}$$

Where, R is the accumulated release (%), V is the sampling volume, V_0 is the initial volume, C_i and C_n are the curcumin concentrations, i and n are the sampling times, and m_{drug} is the mass of curcumin in nanoparticles.

3.7. Inoculation of bacterial infection *on mouse skin*

BALB/c mice were used in all these tests. The animals were anesthetized by ketamine and xylazine and inoculated with *Staphylococcus aureus* and *Pseudomonas aeruginosa* resuspended in PBS for 1h, with about 10^7 CFU/mouse/inoculation. The mice were divided into four groups. Each group was treated once a day with chitosan-TPP nanoparticles, curcumin or curcumin-loaded chitosan-TPP nanoparticles for 3 days respectively. A non-treated group also used as a negative control. They were kept separately with free access to water and food. All the groups were sacrificed 4 days post infection, and the skin tissues were assessed for *Staphylococcus aureus* and *Pseudomonas aeruginosa* infection.

3.8. Statistical Analysis

Statistical analyses were performed using SPSS (Version 21, IBM, USA) software and graphical representations were showed by Excel 2007 (Version 12, Microsoft office, USA) software. Mann-Whitney U test was used to compare different groups and *p* values less than 0.05 were considered statistically significant. The data are presented here as mean ± SD of three independent experiments.

4. Results

4.1. Curcumin-loaded chitosan-TPP nanoparticles properties

Curcumin-loaded chitosan-TPP nanoparticles were prepared and optimized in size, charge and shape. The average diameter of nanoparticles was 160 ± 10 nm and their surface charge was +7 ± 2 mV (Figure 1). Curcumin-loaded chitosan-TPP nanoparticles showed the typical spherical shape (Figures 2 A-B). FTIR spectra of chitosan, curcumin and curcumin-loaded chitosan-TPP nanoparticles are shown in Figure 3. According to the Figure, two characterization peaks (1103 cm^{-1} of

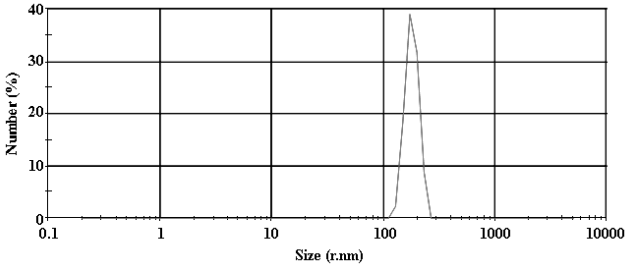

Figure 1. The average diameter of curcumin-loaded chitosan-TPP nanoparticles. The average diameter of these nanoparticles is 160 ± 10 nm

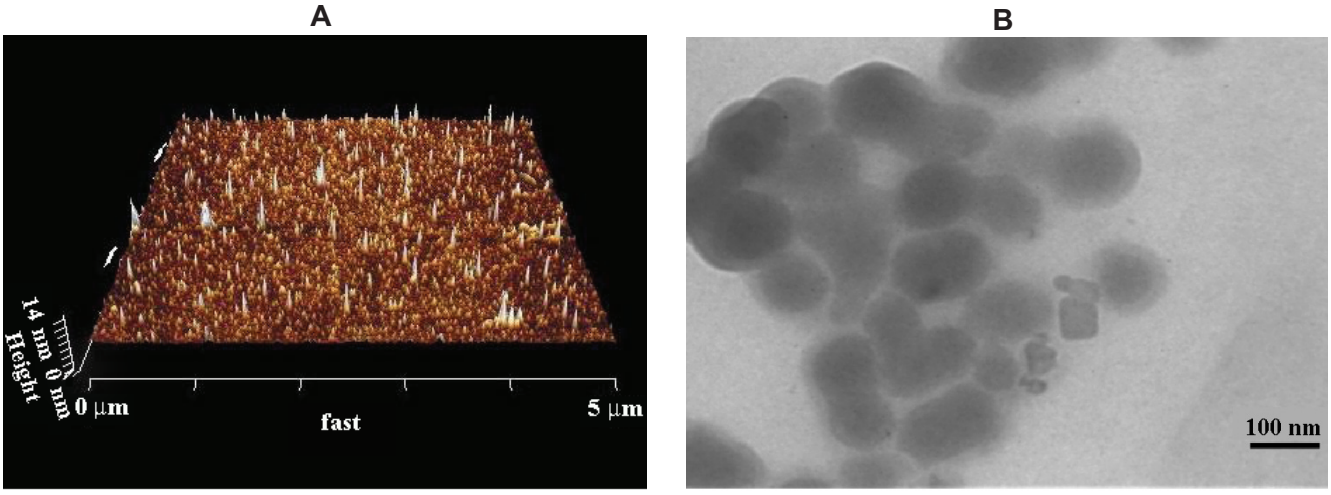

Figure 2. AFM (A) and TEM (B) of curcumin-loaded chitosan-TPP nanoparticles. The average diameter of these nanoparticles is 160 ± 10 nm and their shape is typically spherical

ν (C-O-C) and 1419 cm^{-1} of ν (OH)) existed in the spectrum of curcumin and three characterization peaks (1033 cm^{-1} of ν (C-O-C), 1558 cm^{-1} and 1380 cm^{-1} of ν (NH_2)) existed in the spectrum of chitosan. In comparison with curcumin, a different spectrum was observed for curcumin-loaded chitosan-TPP nanoparticles and new sharp peaks appeared at 2846 cm^{-1} and 1079 cm^{-1}. Also, the 1419 cm^{-1} peak vibration shifted to 1689 cm^{-1}. It can be supposed that the ammonium groups of chitosan were linked with hydroxide groups of curcumin in nanoparticles. The same results had been reported by the previous studies on curcumin loading in chitosan nanoparticles (26, 27).

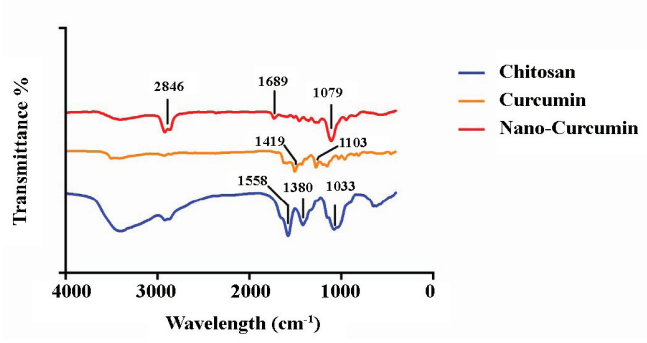

Figure 3. FTIR analysis of curcumin-loaded chitosan-TPP nanoparticles (Nano-curcumin). In comparison with curcumin, a different spectrum was observed for curcumin-loaded chitosan-TPP nanoparticles and new sharp peaks appeared at 2846 cm^{-1} and 1079 cm^{-1}. Also, the 1419 cm^{-1} peak vibration shifted to 1689 cm^{-1}. It could be offered that curcumin was linked with chitosan in nanoparticles

4.2. The encapsulation efficiency of curcumin-loaded chitosan-TPP nanoparticles

After the preparation of curcumin-loaded chitosan-TPP nanoparticles, this nano-formulation were centrifuged and harvested. The amount of curcumin remaining in the supernatant of the solution was then measured by a spectrophotometer. The encapsulation efficiency was determined 75 ± 2% (Table 1).

4.3. The profile of curcumin release from curcumin-loaded chitosan-TPP nanoparticles

According to the release curves, curcumin released from chitosan-TPP nanoparticles over a 96 h period and this time was slower at pH 7.4 as compared to pH 5.4. In comparison with the release profiles of free curcumin, there are similar release profiles at pH 7.4 and 5.4. Under the studied conditions, within 48 h, 41.19% and 82.26% of physically loaded curcumin was released at pH 7.4 and 5.4, respectively and during 72 h, 42.23% and 93.75% of physically loaded curcumin was released at pH 7.4 and 5.4, respectively. After 96 h, 52.41% and 98.91% of physically loaded curcumin was released at pH 7.4 and 5.4, in order (Figure 4).

4.5. The inhibition of bacterial infection by curcumin-loaded chitosan-TPP nanoparticles

The infected mice were treated with curcumin-loaded chitosan-TPP nanoparticles in comparison with the non-treated ones and evaluated for the

Table 1. The encapsulating efficiency (%) of curcumin loaded chitosan-TPP nanoparticles. The most encapsulation efficiency was determined 75 ± 2%

Chitosan concentration mg/mL	Curcumin concentration mg/mL	Encapsulation efficiency (%)
1	0.75	59 ± 1.6
1	1	75 ± 2
1	1.25	71 ± 2.6
1	1.5	67 ± 1.3
1	1.75	63± 1.5
1	2	60± 1.5

expression of bacterial count. The count of *Staphylococcus aureus* was analyzed to explore the effect of curcumin, chitosan-TPP nanoparticles and curcumin-loaded chitosan-TPP nanoparticles on infection. The statistical analyses showed that curcumin-loaded chitosan-TPP nanoparticles significantly decreased the growth of *Staphylococcus aureus* (Mann-Whitney U test; $p<0.05\%$), compared to the control groups (Figure 5). The count of *Pseudomonas aeruginosa* was analyzed to explore the effect of curcumin, chitosan-TPP nanoparticles and curcumin-loaded chitosan-TPP nanoparticles on infection. The statistical analyses showed that curcumin-loaded chitosan-TPP nanoparticles significantly decreased the growth of *Pseudomonas aeruginosa* (Mann-Whitney U test; $p<0.05\%$), compared to the control groups (Figure 6).

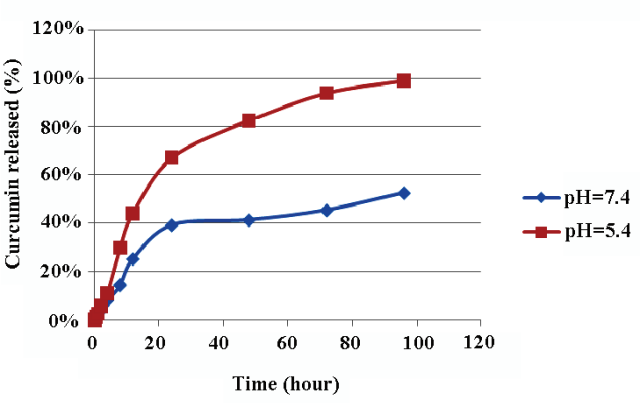

Figure 4. Release curves of curcumin-loaded chitosan-TPP nanoparticles. According to the release curves, curcumin released from chitosan-TPP nanoparticles over a 96 h period and this time was slower at pH 7.4 as compared to pH 5.4

5. Discussion

Curcumin as a potent, biocompatible and bioactive agent with an antimicrobial property has been applied in nanoparticles synthesis (28, 29). In the present study, curcumin was loaded in chitosan-TPP nanoparticles by ionotropic gelation method. AFM and TEM revealed the average diameter of 160 ± 10 nm and a spherical shape for curcumin-loaded chitosan-TPP nanoparticles. By contrast, Das et.al loaded curcumin in chitosan nanoparticles by applying sodium alginate as a cross linker instead of TPP salt. Their nanoparticles size was 100 ± 20 nm with spherical shape (27). Akhtar et.al also produced curcumin-loaded chitosan nanoparticles with size of more than 200 nm while curcumin-loaded chitosan nanoparticles with size of 160 nm were made in our research (30). Here, surface charge of the produced curcumin-loaded chitosan nanoparticles was +7 ± 2 mV whereas the surface charge of chitosan-TPP nanoparticles alone has been reported +25 ± 4 mV. As a result, by loading curcumin the charge of chitosan-TPP nanoparticles decreased (26). After curcumin loading in nanoparticles, curcumin-loaded chitosan nanoparticles got soluble and a transparent solution was obtained. The transparency of nanoparticles solution indicated that curcumin as a hydrophobic material can be emulsified in chitosan-TPP nanoparticles along with applying tween 80 emulsifier. In the present study,

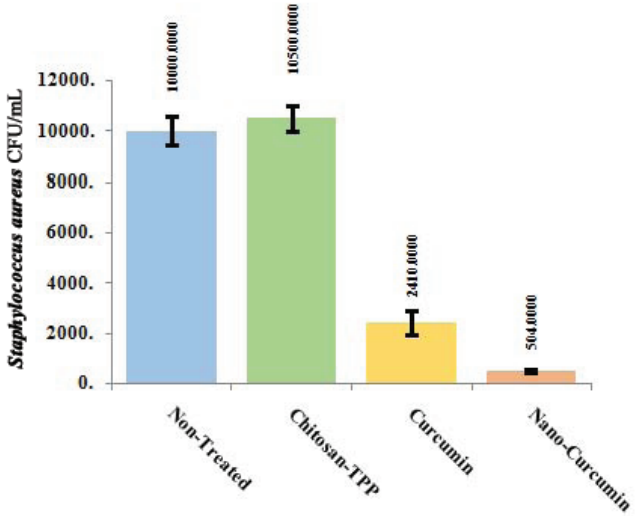

Figure 5. Antibacterial activity of curcumin-loaded chitosan-TPP nanoparticles (Nano-curcumin). Curcumin-loaded chitosan-TPP nanoparticles significantly inhibited *Staphylococcus aureus* infection (Mann-Whitney U test; $p<0.05\%$), compared to chitosan-TPP and curcumin

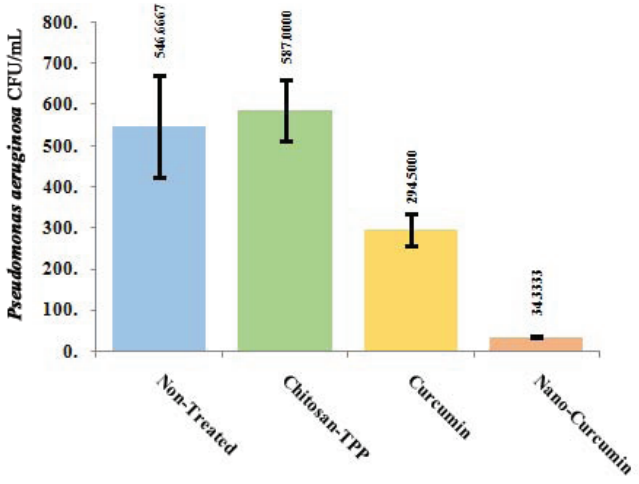

Figure 6. Antibacterial activity of curcumin-loaded chitosan-TPP nanoparticles (Nano-curcumin). Curcumin-loaded chitosan-TPP nanoparticles significantly inhibited *Pseudomonas aeruginosa* infection (Mann-Whitney U test; $p<0.05\%$), compared to the chitosan-TPP and curcumin

the encapsulation efficiency of curcumin in chitosan-TPP nanoparticles was 75 ± 2% while in Das *et al*., study the entrapment efficiency was less than 20% (27). Probably, using TPP cross linker caused to increase of curcumin loading in chitosan-TPP nanoparticles. The peaks characterized by FTIR analysis indicated that chitosan was linked to TPP salt by ammonium groups of chitosan and phosphate groups of TPP (26). Curcumin is more likely to link to chitosan via involving the hydroxide groups of curcumin and ammonium groups of chitosan (27).

To compare with Das *et al*., study in which the kinetic of release showed 80% release of curcumin from nanoparticles, in our survey 52.41% and 98.91% of physically loaded curcumin was released at pH 7.4 and 5.4 within 96 h, respectively. The slower release of curcumin from curcumin-loaded chitosan-TPP nanoparticles at pH 7.4 is perhaps due to chitosan nature as this molecule is more soluble and degradable in acidic pH than neutral and/or alkaline pH (27). For the mentioned results, we used curcumin-loaded chitosan-TPP nanoparticles to suppress both Gram negative and Gram positive bacteria including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This study has shown that curcumin-loaded chitosan-TPP nanoparticles could significantly suppress the progression of *Staphylococcus aureus* and *Pseudomonas aeruginosa* infection on mouse skin, whereas chitosan-TPP by itself could not inhibit bacterial infection. We observed that antimicrobial effect of curcumin-loaded chitosan-TPP nanoparticles is significantly better than individual curcumin. Nanoparticles in drug delivery systems are able to diminish the defects of usual delivery systems (18, 25) and it is probably due to the gradual release of curcumin by nanoparticles in infection areas. Accordingly, combination of chitosan-TPP nanoparticles and curcumin produces a synergy effect on the antibacterial activity that is more than both of them individually. Bhawana et al. have also displayed that curcumin was able to inhibit the bacterial infection. They demonstrated that the cytotoxicity effect of curcumin-loaded chitosan-TPP nanoparticles was greater on Gram-positive bacteria than Gram-negative bacteria (29). Our experiments showed the efficacy and effectiveness of curcumin-loaded chitosan-TPP nanoparticles on both Gram-positive and Gram-negative bacteria including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. De *et al*. have also elucidated antimicrobial activity of curcumin against helicobacter pylori (28). According to Bansal *et al*., research, curcumin was able to inhibit nosocomial infections *caused by Klebsiella pneumonia,* an opportunistic pathogen (31). These studies have been shown antimicrobial effect of curcumin while our study has displayed a higher potency of curcumin-loaded chitosan-TPP nanoparticles rather than the individual curcumin. In another research, Singh *et al*., studied antibacterial properties of curcumin. They declared that curcumin was a potent molecule in the treatment of bacterial infections (1). In agreement with our study, Rai *et al*., suggested that curcumin might be considered as an important antibacterial drug target. In this study, curcumin has been shown to have a potent antibacterial activity against a number of pathogenic bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis and Enterococcus* (32). Also, Wang et al. used microcapsule curcumin against *Staphylococcus aureus, Escherichia coli, Yersinia enterocolitica, Bacillus subtilis and Bacillus cereus*. Therefore, their observation indicated that curcumin induced a spectrum inhibitory effect against all these organisms (2).

To conclude, curcumin-loaded chitosan-TPP nanoparticles can potentially be utilized in drug delivery systems and applied as a strategy to specifically activate antibacterial system at the

same time. This property of curcumin-loaded chitosan-TPP nanoparticles introduces them as a good candidate for drug targeting of bacterial infection including *Staphylococcus aureus* and *Pseudomonas aeruginosa* infection.

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