RESEARCH ARTICLE

Investigation of chitosan nanoparticles durability in combination with antioxidant-antibacterial fraction extracted from Lactobacillus casei and possible increase of antibacterial activity of the fraction in hybrid nanoparticle

Zahra Pourramezan[1*]; Rouha Kasra Kermanshahi[1]; Ali Asghar Katbab[2]

[1]Department of Microbiology,Faculty of Biological Sciences, Alzahra University, Tehran, Iran

[2]Department of Polymer Engineering and Color Technology, Amirkabir University of Technology, Tehran, Iran

ARTICLE INFO

Article History:
Received 27 March 2017
Accepted 28 May 2017
Published 29 May 2017

Keywords:
Chitosan nanoparticles
Ionic gelation
Antioxidant-antibacterial fraction
Lactobacillus casei

ABSTRACT

Objective(s): This study considered the combination of chitosan nanoparticles with antioxidant-antibacterial fraction extracted from Lactobacillus casei and investigation of possible increasing of antibacterial activity of the fraction in hybrid nanoparticle and the effect of the fraction on the stability of chitosan nanoparticles.

Methods: Extraction of Antioxidant antibacterial material from Lactobacillus casei supernatant was done by thin layer chromatography fractionation. For determination of antioxidant and antibacterial activity of fraction, DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and Minimum Inhibition Concentration (MIC) by micro-well dilution method was used, respectively. For chitosan nanoparticles (Cs NPs) formation, the ionic gelation method was used and the ratio of Tripolyphosphate pentasodium (TPP): chitosan was optimized. For Antioxidant fraction loaded chitosan nanoparticles, the fraction is physically incorporated into the chitosan nanoparticles. Particle morphology was monitored by Scanning Electron Microscopy (SEM).

Results: One polar fraction by Rf = 0.03 has a strong antibacterial activity that shows terpenoids characterization. Chitosan nanoparticle loaded antioxidant-antibacterial material has longer life span while compare to Cs-NPs alone. The antioxidant-antibacterial material was released relatively slowly from the CS NPs. the antibacterial trait of the fraction was increased about 8-16 times after combination with Cs NPs.

Conclusions: Combination of Cs NPs with antioxidant-antibacterail fraction isolated from Lactobacillus casei increase the Cs NPs stability and antibacterial activity of the fraction was enhanced considerably, also.

How to cite this article:
Pourramezan Z, Kermanshahi R K, Katbab A A, Investigation of chitosan nanoparticles durability in combination with antioxidant-antibacterial fraction extracted from Lactobacillus casei and possible increase of antibacterial activity of the fraction in hybrid nanoparticle. Nanomed Res J, 2017; 2(2):123-130. DOI: 10.22034/nmrj.2017.62889.1065

INTRODUCTION

Today, nanotechnology has found a wide range of applications in the pharmaceutical industry [1]. Because of the new promotion in nanotechnology, it is now achievable to create drug nanoparticles that can be used in different ways [2]. Over the last years, chitosan micro- and nano-particles has considered as an attractive compound in the biomedical and pharmaceutical fields [3-5] including wound dressings [4], prolonged or controlled release drug delivery systems [3], blood anticoagulants [6], and space filling implants [7]. Chitosan is a copolymer construct from N-acetyl glucosamine and glucosamine units [8]. It has

* Corresponding Author Email: *z.pourramezan@gmail.com*

antibacterial and anti-fungal properties [9-11]. Furthermore, chitosan has been recently used as an adjunctive for an antimicrobial drug in order to raise its pharmacological activity [12]. In spite of the great potential of this polymer, its poor stability over time made chitosan-based systems not applicable as final pharmaceutical products [13]. Upon storage, chitosan is subjected to gradual chain deterioration followed by elimination of its functional groups which as a result leads to irreversible lack of its physicochemical properties. Both intrinsic (molecular weight, the degree of deacetylation, purity, and moisture level) and extrinsic factors (thermal processing, environmental storage conditions, sterilization, and processing involving acidic dissolution) are acknowledged as critical parameters influencing the stability of the chitosan-based formulations. To boost chitosan stability, several strategies (addition of the stabilizing agent during the preparation process, use of ionic or chemical crosslinkers, and blending with hydrophilic polymer) have also been reported [14]. As a result, scientists have put an attempt into improving the stability characteristics of chitosan products [13].

Jang and Lee (2008) showed that by adding antioxidant agent such as L-ascorbic acid to chitosan nanoparticles, the stability of NPs was enhanced [15]. In this study, the addition of stabilizing agent like antioxidant agent was used for elevating the stability of chitosan nanoparticles during the preparation process. For this purpose, the antioxidant material extracted from Lactobacillus spp. has been suggested. Lactobacillus spp. are important members of the healthy human microbiota that was shown to have antioxidative properties [16]. We reported the antioxidant fraction with antibacterial activity extracted from Lactobacillus casei strain K1C (Accession number KU954559) in a previous study (unpublished data). The purpose of this study was to compare the antibacterial activity of antioxidant fraction alone and after mixing with chitosan nanoparticle and evaluate the life span of chitosan with and without the isolated fraction.

MATERIALS AND METHODS

Mueller–Hinton broth (MHB) (Merck, Germany), Chitosan (DD > 75%, MW = 120kDa, Merck, Germany), Tripolyphosphate pentasodium (TPP) (Merck, Germany), acetic acid (Merck, Germany) sodium hydroxide (Merck, Germany), phosphate-buffered saline (PBS, Sigma Aldrich), silica gel 60 F254 plate (Merck, Darmstadt, Germany), methanol (Merck, Germany), chloroform (Merck, Germany), DPPH (Sigma, USA). Kefir was collected in Tehran province, Iran. PMM5 broth was prepared according to the method of Wegkamp et al. (2009) [17]. All other materials were purchased from Sigma, USA.

Extraction of Antioxidant antibacterial material from Lactobacillus casei supernatant

Lactobacillus casei strain K1C that was isolated from Kefir was cultured in (Plantarum minimum medium) PMM5 broth [17] anaerobically at 37°C for 48h. Cell-free culture supernatants of Lactobacillus casei were prepared according to the method described by Saadatzadeh et al. [18]. Briefly, the medium was centrifuged at 4000 × g for 15min. The supernatant was passed through sterile 0.22 µm pore-size filter (Sartorius, Germany). Freeze-dried/Methanol extracts of filtrate were dried and kept at the tight container in 4°C.

A fixed concentration and amount of methanol extract (10 µL of 10 mg/ml) was spotted on a precoated silica gel 60 F254 plate (Merck, Darmstadt, Germany). The plate was developed with methanol: chloroform (3:2) solvent system. The developed plates were air dried and observed under ultra violet (UV) and visible light. For the antioxidant capacity screening, the developed air dried plate was soaked in methanolic solution of 0.1% DPPH antioxidant reagent, and the plates were air-dried. The active antioxidant components were detected as yellowish spots on purple background on the air-dried TLC plates. The Rf values of all detected active antioxidant constituents were determined [19].

Fractions with the same Rf value were collected from 20 plates. The collected sample was dissolved in HPLC grade methanol and centrifuged at 12000rpm for 15min (Hettich Universal, Germany) in order to remove silica, then the methanolic extracts were dried up in rotary (Heidolph, Germany) below 40°C. The residue was weighed to determine the amount of each fraction for antibacterial assay [20].

Partial characterization of the active component

The antioxidative semi-purified fractions extracted from TLC of methanol extract of PMM5 supernatant after 48 h fermentation by Lactobacillus casei strain K1C were tested for the presence or absence of different compounds by using the standard methods of Jayashree (2013). Briefly, for detection of carbohydrate, peptides, phenol and polyphenol, phenols and tannins, alkaline and terpenoids compounds in the TLC, the following reagents were used respectively; Benedict's reagents, ninhydrin, Folin–Ciocalteu, $FeCl_3$ 2%, NaOH 2% and chloroform-H_2SO_4 [21].

Chitosan Nanoparticles Preparation

Chitosan nanoparticles (CS NPs) were prepared according to the modified method described by Ibrahim et al. [22]. For optimization of nanoparticle preparation, different acetic acid concentration, pH and the ratio of Tripolyphosphate pentasodium (TPP): chitosan was used each time. Briefly, a solution of a chitosan was prepared at a concentration of 0.1-1.5% (w/v) in acetic acid (0.5%) and the pH of this solution was adjusted to 4-5.5 and was continuously stirring at 500 × g and room temperature for 24 h. chitosan solution was added drop wise to the TPP solution (0.1-1 %) while continuously stirring (IKA RH basic 2, Germany). The prepared nanoparticle was undergone 20W sonification for 9min. The nanoparticles were centrifuged at 16000×g for 30min, divided into aliquots which were used for use in chitosan stability experiments.

Preparation of Chitosan-antioxidant Nanoparticles with antibacterial activities

Antioxidant loaded chitosan nanoparticles are prepared in the equal way as CS NPs except antioxidant-antibacterial fraction is pre-dissolved in the TPP solution at a concentration of 1mg/ml [22].

Determination of particle morphology by scanning electron microscopy

Studies of the particle morphology were conducted by scanning electron microscopy (SEM) (Tescan, Vega3, USA). Dried particles were taken in a piece of black tape and attached to the sample holder. Particle morphology was examined under low vacuum.

Yield of Chitosan Nanoparticles %

The percentage yields of chitosan nanoparticles were calculated from the weight of dried nanoparticles recovered and sum of initial dry weight of starting material as the following equilibrium [23]:

$$Percent\ Yield = \frac{the\ amount\ of\ nanoparticles\ obtained\ (g)}{the\ theoretical\ amount\ (g)} \times 100$$

Durability of CS NPs

Freshly prepared CS NPs were centrifuged at 16000×g for 30min prior to storing. After ultracentrifugation, the obtained pellets were suspended in phosphate buffered saline (PBS) pH7.4. The weight loss were measured at predetermined storage time durations (0,1,2,3,4 and 5 weeks), and at 37°C. Samples were removed from the PBS at specific time intervals and washed with distilled water. The dried weight of nanoparticles that was kept overnight in an oven at 45°C was calculated according to the following formula:

$$\%\ Weight\ loss = \left[\frac{(Wi - W_t)}{Wi}\right] \times 100$$

Where, W_i is the initial mass and W_t is the remaining mass at time t. All results were the average of two replicates [24].

In Vitro Drug Release Study

The release of antibacterial material from CS NPs was determined by incubating the nanoparticles in 5 ml of pH 7.4 phosphate buffer solution (PBS) at 37°C. Antibacterial fraction loaded CS NPs were suspended in Tris-HCl buffer solution (pH 7.4) and placed in a shaker incubator with a stirring speed of 100 ×g at 37°C (Pars Azma, Iran) for 48 h at 37°C. At predetermined time intervals (24, 48, 72, 96, and 120h), samples were centrifuged at 15000 × g for 30 min at 10°C. Then, the supernatant was poured out. The amount of released antibacterial fraction in the supernatant was analyzed by a UV-Vis spectrophotometer (Labomed, USA) at a wavelength of 250nm at which the antibacterial fraction has the highest absorbance [25]. Triplicate samples were analyzed for each measurement.

Antibacterial assay

The Minimum Inhibition Concentration (MIC) values of the isolated antibacterial fraction, CS

NPs, and antibacterial loaded CS NPs against the pathogenic bacteria were determined using the micro-well dilution method according to the CLSI protocol [26]. While, in MIC assay, the sample tested were further diluted ranging from 1000 to 2 µg/ml.

Escherichia coli ATCC 11303 and Staphylococcus aureus ATCC 6538 were selected as indicator pathogenic bacteria because they are the most frequent bacteria in the wound infection and represent Gram negative and Gram positive bacteria, respectively. All experiments were done in triplicate and chloramphenicol was used as standard antibiotic.

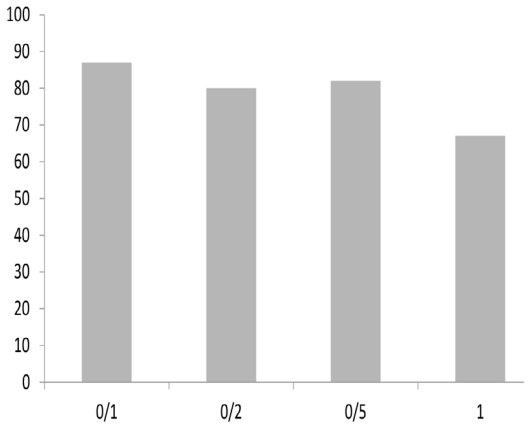

Fig. 1. Yield of CS-NPs % (y-axis) prepared by using of 0.5 mg/ml TPP and different chitosan concentration (mg/ml) (x-axis).

RESULTS AND DISCUSSION

Chitosan nanoparticles were used as drug delivery carriers due to it offers many advantages. In addition, nano-sized particles and the absorption increasing effect displayed ability to improve drug bioavailability [27-30].

During CS NPs preparation, after drop wise addition of the chitosan solution to the stirring TPP solution (0.1-1 %), three various suspension were visually analyzed that included: opalescent suspension, clear solution, and aggregates. The opalescent suspension, which correlated to a suspension of very tiny particles, is illustrated in Figure 1 as a combination of different CS and the TPP concentrations. This opalescent suspension was achieved in a final concentration of CS and TPP between 0.1–1 mg/ml and 0.5 mg/ml, respectively that different sizes of the nanoparticles could be attained (Table 1) and yield of CS-NPs prepared by using of 0.5 mg/ml TPP (Table 2) and different chitosan concentration (mg/ml) were shown at Figure 1.

SEM image of CS-NPs and antibacterial-loaded chitosan nanoparticle was demonstrated at Fig 2.

Xu and Du have studied different formulations of chitosan nanoparticles produced by the ionic gelation of TPP and CS. According to their TEM results, the diameters of Cs- NPs were ranged between 20 and 200 nm. Pan et al. reported that the particle size of chitosan nanoparticles was in the range of 250-400 nm that is in agreement with our results that was less than 500nm [31]. Previous studies have shown that the loaded nanoparticles would normally produce a larger size than the empty ones and the size of below 500 nm is suitable [32].

Cs NPs formed by ionic gelation have weak mechanical strength thus, limiting their usage in

Table 1. Optimization of Cs: TPP concentrations for the formation of CS-NPs.

TPP (mg/ml) / Cs (mg/ml)	0.1	0.5	1
0.1, 0.2, 0.5 , 1	Clear solution	opalescent suspension	aggregates

Table 2. Yield of Chitosan Nanoparticles% were prepared by using of TPP concentration of 0.5 mg/ml

chitosan concentration (mg/ml)	Yield of CS-NPs %
0/1	87
0/2	80
0/5	82
1	67

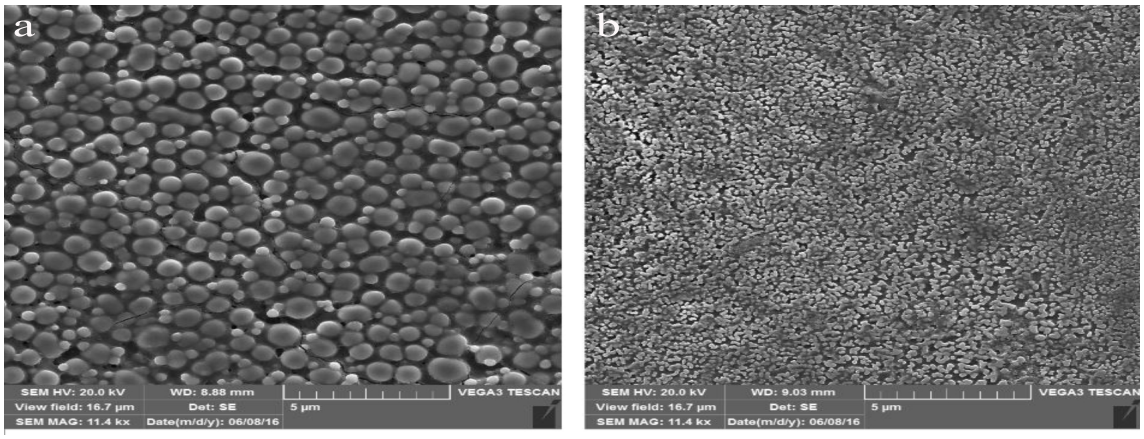

Fig. 2. SEM image of (a) Cs NPs (chitosan 1 mg/ml: TPP 0.5 mg/ml)
(b) antibacterial- loaded Cs NPs (chitosan 0.1 mg/ml: TPP 0.5 mg/ml)

drug delivery [33]. To improve chitosan stability, several strategies including addition of the stabilizing agent like antioxidant material during the preparation process were used.

Drug loading in nanoparticulate systems can be conducted done by two methods, i.e., after the production of particles (incubation) and during the formation of particles (incorporation). In these two systems, drug is took up onto the surface or physically inserted into the matrix, respectively. Maximum drug loading can be achieved by incorporating the drug during the formation of particles [34]. In the current study, three antioxidant fraction (Rf = 0.03, 0.2, 0.34) were isolated from L. casei strain K1C that only one fraction (F1, Rf = 0.03) show the characterization of terpenoids components and antibacterial traits against pathogenic bacteria that was incorporated and embedded into Cs NPs during the preparation of particles (incorporation). Although, the particle size was expected to increase when antibacterial fraction was successfully being loaded into nanoparticles, But by using different ration of Cs: TPP (1:5), the size of loaded CS NPs was smaller than CS NPs alone (with Cs: TPP, 1: 0.5) as it shown in Fig. 2. This result demonstrates that the Cs: TPP ratio has an important effect on nanoparticle sizes. In summary, CS and TPP concentrations were the parameters controlling particle size of CS NPs that is in agreement with the results of Esmaeilzadeh-Gharedaghi et al. [35]. Nanoparticles less than 500 nm could be obtained at TPP: CS weight ratio of 5:1 at pH 4.

According to the results, antibacterial- loaded Cs NPs at week 5 show the same weight loss of Cs NPs at week 1 (Fig. 3).

Storage temperature and suspending medium were found to be the factors that could influence the stability of CS NPs. Katas et al. (2013) stated that CS NPs should not be stored at room temperature as they are susceptible to degradation and CS NPs were more stable when stored at 4°C [36]. CS NPs were labile at 37°C. In addition, CS NPs had better stability in distilled water than in PBS which might be due to hydrogen bonds that formed between ionizable groups of water molecules and CS NPs [36]. So, in this study, for stability investigation of CS NPs and antibacterial- loaded Cs NPs, incubation was done in PBS at 37°C.

The antioxidant-antibacterial material was slowly released from 20h up to 120h, resulting in a release of less than 20% (Fig. 4).

According to the results of Sunil et al. (2004) Chitosan microparticles that were prepared at lower pH or higher concentration of TPP solution resulted in a slower release of the drug [34]. In addition, with a decreasing molecular weight and concentration of CS solution, the drug release increased. But according to the results of present study, the higher concentration of TPP solution and decreasing concentration of CS solution resulted in a relatively slow release of the drug.

In this study, slow rate of drug release arises from the stability of this carrier due to loaded antioxidant. Antibacterial assay of CS NPs, antibacterial fraction, and antibacterial-loaded Cs NPs were shown in

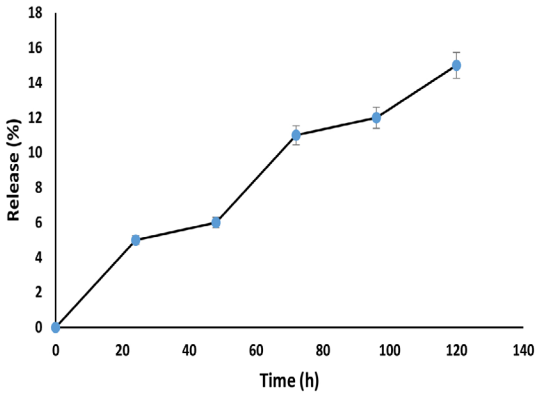

Fig. 3. Weight loss (%) of chitosan and chitosan-antibacterial nanoparticles during 5 weeks

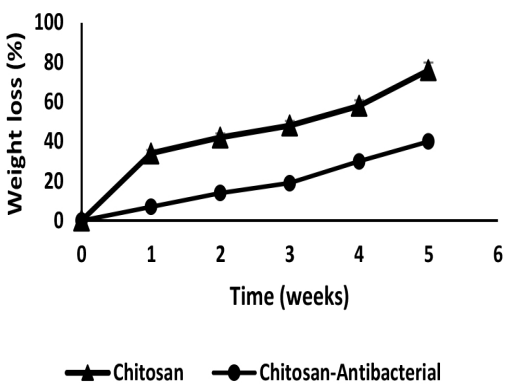

Fig. 4. Release rate (%) of antioxidant-antibacterial fraction in PBS at 37°C under continuously shaking for 120h incubation.

Table 3. The MIC (µg/ml) of Chloramphenicol for Staphylococcus aureus ATCC 6538 and Escherichia coli ATCC 11303 was 2 and 0.5, respectively.

In this study, the antibacterial fraction was added to CS solution before cross-linking, but it showed relatively high antibacterial activity (MIC 60 µg/ml), so, it can be concluded that by using of incubation with the pre-formed NPs, the antibacterial activity of fraction-loaded Cs NPs can be increased.

One of the antibacterial mechanism of chitosan is explained that chitosan binds to the negatively charged bacterial surface damaging the cell membrane and changing its permeability. Therefore, causing cell death [37, 38] According to the results of present research, Cs NPs shows higher antibacterial activity against Gram positive bacteria than Gram-negative bacteria, this is due to their different cell walls. the bilayer structure of the outer membrane in gram negative bacteria is a potential barrier against foreign molecules [37]. The antibacterial-loaded Cs NPs resulted in the least MIC against E. coli (60µg/ml) that shows the enhancement of antibacterial trait of antibacterial fraction (MIC 500 µg/ml) embedded into Cs NPs (MIC 2500µg/ml).

CONCLUSIONS

Drug delivery systems are one of the extensively investigated fields utilizing chitosan because of the efficiency of chitosan for entrapping a particular drug and its ability for controlled drug release. Despite the great potential of using chitosan in drug delivery or tissue engineering systems, its poor long-term stability is a substantial drawback in the scaling-up of chitosan pharmaceutical applications. To improve chitosan stability, the antioxidant fraction with antibacterial traits was added to Cs NPs during the preparation process. The combination of Cs NPs with antioxidant-antibacterial fraction isolated from Lactobacillus casei increase the Cs NPs stability and antibacterial activity of the fraction was enhanced considerably too. This study has shown that the shelf life, and antibacterial traits of CS NPs can be enhanced by incorporation into bioactive fractions isolated from Lactobacillus casie and antibacterial activity of the

Table 3. Antibacterial assay (MIC and MBC) of CS NPs, antibacterial fraction and antibacterial-loaded Cs NPs.

Pathogenic bacteria	CS NPs MIC (µg/ml)		Antibacterial fraction		CS-antibacterial NPs	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
Escherichia coli ATCC 11303	2500	4500	500	1000	60	115
Staphylococcus aureus ATCC 6538	1250	2500	1000	1000	60	115

fraction was increased by incorporation into CS NPs, too.

Also, the ideal conditions such as pH and chitosan/TPP ratio were optimized during the nanoparticle formation period. In conclusion, the isolated antioxidant-antibacterial fraction can be used to change the chemical stability and biodegradation rate of chitosan nanoparticles for possible application as a wound dressing with elevated antibacterial activity with higher life span.

ACKNOWLEDGEMENTS

This work has been financed from the grant of the Alzahra University of Iran.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

1. Ober CA, Gupta RB. Nanoparticle Technology for Drug Delivery. Ide@s CONCYTEG. 2011;6(72):714-26.
2. Jain KK. Ethical, Safety, and Regulatory Issues of Nanomedicine. The Handbook of Nanomedicine: Springer; 2008. p. 329-52.
3. SzymaSka E, Winnicka K. Preparation and in vitro evaluation of chitosan microgranules with clotrimazole. Acta Pol Pharm Drug Res. 2012;69(3):509-13.
4. Hurler J, Škalko-Basnet N. Potentials of chitosan-based delivery systems in wound therapy: Bioadhesion study. J Funct Biomater. 2012;3(1):37-48.
5. Venkatesan J, Bhatnagar I, Kim S-K. Chitosan-alginate biocomposite containing fucoidan for bone tissue engineering. Mar drugs. 2014;12(1):300-16.
6. Okamoto Y, Yano R, Miyatake K, Tomohiro I, Shigemasa Y, Minami S. Effects of chitin and chitosan on blood coagulation. Carbohydr Polym. 2003;53(3):337-42.
7. Hoemann C, Sun J, Legare A, McKee M, Buschmann M. Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle. Osteoarthritis cartilage. 2005;13(4):318-29.
8. Chattopadhyay D, Inamdar M. Improvement in properties of cotton fabric through synthesized nano-chitosan application. JFTR. 2013;38(1):14-21.
9. Benhabiles M, Salah R, Lounici H, Drouiche N, Goosen M, Mameri N. Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. Food hydrocolloids. 2012;29(1):48-56.
10. Lahmer RA, Williams AP, Townsend S, Baker S, Jones DL. Antibacterial action of chitosan-arginine against Escherichia coli O157 in chicken juice. Food Control. 2012;26(1):206-11.
11. Cruz-Romero M, Murphy T, Morris M, Cummins E, Kerry J. Antimicrobial activity of chitosan, organic acids and nano-sized solubilisates for potential use in smart antimicrobially-active packaging for potential food applications. Food Control. 2013;34(2):393-7.
12. Szymańska E, Winnicka K, Wieczorek P, Sacha PT, Tryniszewska EA. Influence of Unmodified and β-Glycerophosphate Cross-Linked Chitosan on Anti-Candida Activity of Clotrimazole in Semi-Solid Delivery Systems. Int J Mol Sci. 2014;15(10):17765-77.
13. Mostafa Amin D, Adel Zak E-S, Mohamed Mohamed A-H, Dina Mohamed Diaa B. Thermal stability and degradation of chitosan modified by cinnamic acid. OJPChem. 2012;2012.
14. Szymańska E, Winnicka K. Stability of chitosan—a challenge for pharmaceutical and biomedical applications. Mar drugs. 2015;13(4):1819-46.
15. Jang K-I, Lee HG. Stability of chitosan nanoparticles for L-ascorbic acid during heat treatment in aqueous solution. J Agric Food Chem. 2008;56(6):1936-41.
16. Xing J, Wang G, Zhang Q, Liu X, Gu Z, Zhang H, et al. Determining Antioxidant Activities of Lactobacilli Cell-Free Supernatants by Cellular Antioxidant Assay: A Comparison with Traditional Methods. PLOS ONE. 2015;10(3):e0119058.
17. Wegkamp A, Teusink B, De Vos W, Smid E. Development of a minimal growth medium for Lactobacillus plantarum. Lett Appl Microbiol. 2010;50(1):57-64.
18. Saadatzadeh A, Fazeli MR, Jamalifar H, Dinarvand R. Probiotic properties of Lyophilized cell free extract of Lactobacillus casei. Jundishapur J Nat Pharm Prod. 2013;8(3):131-7.
19. Jaime L, Mendiola JA, Herrero M, Soler-Rivas C, Santoyo S, Señorans FJ, et al. Separation and characterization of antioxidants from Spirulina platensis microalga combining pressurized liquid extraction, TLC, and HPLC-DAD. J Sep Sci. 2005;28(16):2111-9.
20. Arullappan S, Rajamanickam P, Thevar N, Narayanasamy D, Yee HY, Kaur P, et al. Cytotoxic effect and antioxidant activity of bioassay-guided fractions from Solanum nigrum extracts. Trop J Pharm Res. 2015;14(7):1199-205.
21. Jayashree D. Phytochemicals analysis and TLC finger printing Of methanolic extracts of three medicinal plants. Int Res J Pharm. 2013;4(6):123-1236.
22. Ibrahim HM, El-Bisi MK, Taha GM, El-Alfy EA. Chitosan nanoparticles loaded antibiotics as drug delivery biomaterial. J App Pharm Sci. 2015;5(10): 085-090.
23. Sailaja AK. Formulation and evaluation studies of BSA loaded chitosan nanoparticles by polymerization technique. Int J Adv Pharm. 2016;5(3):66-75.
24. Rivero S, García M, Pinotti A. Physical and chemical

treatments on chitosan matrix to modify film properties and kinetics of biodegradation. Journal of Materials Physics and Chemistry. 2013;1(3):51-7.

25.Zuluaga F SDJB. Evaluation of Biocompatibility of Chitosan Films from the Mycelium of Aspergillus niger in Connective Tissue of Rattus norvegicus. J Mol Genet Med. 2015;09(03).

26.Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. 2016;6(2):71-9.

27.Liu L, Dong X, Zhu D, Song L, Zhang H, Leng XG. TAT-LHRH conjugated low molecular weight chitosan as a gene carrier specific for hepatocellular carcinoma cells. Int J Nanomedicine. 2014;9:2879.

28.Lu Y, Cheng D, Lu S, Huang F, Li G. Preparation of quaternary ammonium salt of chitosan nanoparticles and their textile properties on Antheraea pernyi silk modification. Text Res J. 2014;84(19):2115-24.

29.Nascimento AV, Singh A, Bousbaa H, Ferreira D, Sarmento B, Amiji MM. Mad2 checkpoint gene silencing using epidermal growth factor receptor-targeted chitosan nanoparticles in non-small cell lung cancer model. Mol Pharm. 2014;11(10):3515.

30.Ragelle H, Riva R, Vandermeulen G, Naeye B, Pourcelle V, Le Duff C, et al. Chitosan nanoparticles for siRNA delivery: optimizing formulation to increase stability and efficiency. J Control Release. 2014;176:54-63.

31.Pan Y, Li Y-j, Zhao H-y, Zheng J-m, Xu H, Wei G, et al. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int J Pharm. 2002;249(1):139-47.

32.Gan Q, Wang T. Chitosan nanoparticle as protein delivery carrier—systematic examination of fabrication conditions for efficient loading and release. Colloids and Surfaces B: Biointerfaces. 2007;59(1):24-34.

33.Mitra A, Dey B. Chitosan microspheres in novel drug delivery systems. Indian J Pharm Sci. 2011;73(4):355.

34.Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro-and nanoparticles in drug delivery. J Control Release. 2004;100(1):5-28.

35.Esmaeilzadeh-Gharedaghi E, Faramarzi MA, Amini MA, Rouholamini Najafabadi A, Rezayat SM, Amani A. Effects of processing parameters on particle size of ultrasound prepared chitosan nanoparticles: An Artificial Neural Networks Study. Pharm Dev Technol. 2012;17(5):638-47.

36.Katas H, Raja MAG, Lam KL. Development of chitosan nanoparticles as a stable drug delivery system for protein/siRNA. Int J Biomate. 2013;2013.

37.Abou-Zeid N, Waly A, Kandile N, Rushdy A, El-Sheikh M, Ibrahim H. Preparation, characterization and antibacterial properties of cyanoethylchitosan/cellulose acetate polymer blended films. Carbohydr Polym. 2011;84(1):223-30.

38.Avadi M, Sadeghi A, Tahzibi A, Bayati K, Pouladzadeh M, Zohuriaan-Mehr M, et al. Diethylmethyl chitosan as an antimicrobial agent: Synthesis, characterization and antibacterial effects. Eur Polym J. 2004;40(7):1355-61.