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Research Article

In Vitro Antioxidant Activity of Chitosan Aqueous Solution: Effect of Salt Form

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Abstract

Purpose: *To investigate the effect of salt form on the antioxidant activities of chitosan aqueous solution.*
Methods: *The antioxidant activities of chitosan acetate (CS-acetate), chitosan hydroxybenzotriazole (CS-HOBt), chitosan thiamine pyrophosphate (CS-TPP) and chitosan ethylenediaminetetraacetic acid (CS-EDTA) solution were determined employing various established in vitro system such as superoxide and hydroxyl radicals scavenging, metal ion chelating and reducing power. Their chemical structures were characterized by nuclear magnetic resonance (NMR) and Fourier transform infrared spectrophotometry (FT-IR).*
Results: *NMR and FT-IR show confirmed formation of chitosan salts. The 50 % inhibition concentration (IC_{50}) of superoxide and hydroxyl radicals was 0.349 – 1.34 and 0.34 – 1.54 mg/mL, respectively. Among the salt forms, CS-acetate (IC_{50} = 0.349 mg/mL) showed the highest superoxide radical scavenging effect while CS-HOBt (IC_{50} = 0.34 mg/mL) showed the highest hydroxyl radical scavenging effect. With regard to metal ion chelating activity, CS-EDTA showed the highest chelating activity (approx 100 % at 1 mg/mL) while the others showed 20 % activity at a concentration of 1 mg/mL. The results for reducing power indicate that CS-TPP had the highest reducing power.*
Conclusion: *The results indicate that antioxidant activity varied with the salt form. Thus, CS salts may be used as a source of antioxidants for pharmaceutical applications.*

Keywords: *Chitosan, Antioxidant, Hydroxybenzotriazole, Thiamine pyrophosphate, Ethylenediaminetetraacetic acid*

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INTRODUCTION

Chitosan (CS) is a natural copolymer of D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) and is produced by alkaline deacetylation of chitin. It is insoluble at neutral and alkaline pH but is soluble in acidic media. It is also biodegradable, biocompatible, and non-toxic and, therefore, has been employed in biomedical applications such as tissue engineering, wound healing and drug delivery [1-3].

Recently, the antioxidant activities of CS and its derivatives were investigated since CS chains have active hydroxyl and amino groups that can react with free radicals [4]. The derivative studied include water-soluble disaccharide chitosan [5], carboxymethyl chitosan [6], sulfated chitosan [7,8], quaternized chitosan [9], sulfanilamide derivatives of chitosan [10], chitosan crab shells [11], chitosan-N-2-hydroxypropyl trimethyl ammonium chloride [12], 2-[phenylhydrazine -thiosemicarbazone] – chitosan [13] and dietary chitosan [14].

Although CS salts such as hydroxybenzo-triazole (HOBt), thiamine pyrophosphate (TPP) and ethylenediaminetetraacetic acid (EDTA) have been investigated for their biomedical applications, however, their antioxidant activities have not yet been investigated. HOBt is an organic compound often used as a racemization suppressor and is popular for its ability to improve yields in peptide synthesis [15]. Due to the hydroxyl groups presented in HOBt, the molecule can form a salt with the amine groups of CS, thus improving CS water solubility and allowing CS to be dissolved in water. We have previously prepared CS-HOBt for use in nucleic acid delivery [16] as well as CS-HOBt/polyvinyl alcohol blend of biodegradable nanofibers intended for drug delivery or tissue engineering applications [17].

TPP is a thiamine derivative. It plays an essential role as cofactor in key reactions in carbohydrate metabolism [18]. Due to the phosphate groups of TPP, the molecular can salt form with amine groups of CS, helps to improve CS water solubility [18]. The amine groups of TPP, especially at the nitrogen atom (N) of thiazolium can be deprotonated and are always positive even at physiological pH [18]. CS-TPP has been successfully prepared as a novel carrier for siRNA delivery [19]. EDTA is a well-established metal chelator and is soluble at alkaline pH. It has been used to form a complex with CS due to the decreased positive charge of the latter. CS can be rendered readily soluble in water with EDTA due to the carboxyl groups in EDTA structure. CS-EDTA conjugate has been successfully used tp prepare a nanoparticulate gene delivery system [20] and hydrogel films for transbuccal delivery [21].

Therefore, the purpose of this study was to prepare CS-HOBt, CS-TPP and CS-EDTA, and characterize their chemical structures by Fourier transform infrared spectrophotometer (FT-IR) and nuclear magnetic resonance (NMR), as well as evaluate their antioxidant activities.

EXPERIMENTAL

Materials

Chitosan low molecular weight (degree of deacethylation 0.85, MW 110 kDa), hydroxybenzotriazole (HOBt), ethylenedia-minetetraacetic acid (EDTA), thiamine pyrophosphate (TPP), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), ferrozine, nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), and deoxyribose (DR) were all purchased from Sigma-Aldrich Chemical Company, USA. All other reagents and solvents were commercially procured and of analytical grade.

Preparation of chitosan solutions

Aqueous CS solutions were prepared by dissolving CS with HOBt, TPP or EDTA at different weight ratio 1:1, 1:1 and 2:1, respectively. Briefly, HOBt (10 mg), TPP (10 mg) or EDTA (5 mg) was dissolved together with CS (10 mg) in 10 mL of distilled water and vigorously stirred with a magnetic stirrer at ambient temperature until the solution became clear. CS-acetate was prepared by dissolving CS (10 mg) in 10 mL of 1 %v/v acetic acid.

Characterization of chitosan salts

The chemical structure of chitosan salts was characterized using Fourier transform infrared spectrophotometer (FTIR, Nicolet 4700, Becthai, USA). The spectra were obtained by accumulating 32 scans within the range 400 – 4000 cm^{-1}. A ^{1}H-nuclear magnetic resonance spectrometer (NMR, Bruker 300 MHz) was used to investigate the chemical integrity of the chitosan salts (sample weight: 5 mg) using deuterated water (D_2O) as solvent.

Superoxide radical scavenging assay

The superoxide scavenging ability of the CS salts was investigated by the method of Nishikimi et al [22]. The reaction mixture, comprising in each case, CS solution (0.1 - 2 mg/mL), PMS (30 μM), NADH (338 μM) and NBT (72 μM) in phosphate buffer (0.1M pH 7.4), was incubated at room temperature for 5 min and the absorbance measured spectrophotmetrically (Agilent model 8453 E, Germany) at 560 nm against a blank (water). The sample without chitosan salts was used as control. Their scavenging activity was calculated using Eq 1.

Scavenging activity (%) = (1 – As/Ac) x 100 … (1)

where As and Ac are the absorbance of the test sample and control, respectively.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging of the CS salts was investigated by the method of Halliwell et al [23]. CS solutions (0.1 - 2 mg/mL) was incubated with deoxyribose (3.75 mM), H_2O_2 (1mM), $FeCl_3$ (100 μM), EDTA (100 μM) and ascorbic acid (100 μM) in potassium phosphate buffer (20 mM, pH7.4) for 60 min at 37 ^{o}C in a tube. The reaction was terminated by adding 1 mL of TBA (1 %w/v) and 1mL of TCA (2 %w/v), and then heating the mixture in a boiling water bath for 15 min. The contents were cooled and the absorbance of the mixture was measured spectrophotometrically at 535 nm against a blank. The sample without chitosan salts was used as control. Increase in the absorbance of the reaction mixture indicates that oxidation of deoxyribose is increased. Scavenging activity was determined as in Eq 2.

Scavenging activity (%) = (1 – As/Ac) x 100 … (2)

where As and Ac are the absorbance of the test sample and control, respectively.

Metal ion chelating assay

The ferrous ion-chelating potential of the CS salts was investigated according to the method of Decker & Welch [24]. The ferrous ion-chelating ability was monitored by the absorbance of the ferrous iron-ferrozine complex at 562 nm. Briefly, the reaction mixture comprised of the CS solution with varying concentrations, $FeCl_2$ (2 mM) and ferrozine (5 mM), and adjusted to a total volume of 0.8 mL with water, shaken well and incubated for 10 min at room temperature. The absorbance was then measured spectrophotometrically at 562 nm. The ability of CS salts to chelate ferrous ion was calculated using Eq 3.

Chelating activity (%) = (1 – As/Ac) x 100 …. (3)

where As and Ac are the absorbance of the test sample and control, respectively.

Evaluation of reducing power

The reducing power of the salts was determined according to the method of Oyaizu [25]. Each CS solution (0.1 - 2 mg/mL, 2.5 mL) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1 % potassium ferricyanide, and the mixture incubated at 50 °C for 20 min. Thereafter, 2.5 mL of 10 % trichloroacetic acid was added and the mixture centrifuged at 200 g for 10 min. The upper layer (5 mL) was mixed with 5 mL of deionized water and 1 mL of 0.1 % ferric chloride, and the absorbance measured spectrophotometrically at 700 nm against a blank. A higher absorbance indicates a higher reducing power. The sample without chitosan salts was used as control.

Statistical analysis

Data were obtained in triplicate and expressed as the mean ± standard deviation (SD). The data were subjected to analysis of variance (ANOVA) using SPSS software (version 11). The significance level was set at $p < 0.05$.

RESULTS

Characteristics of the chitosan salts

The pure chitosan powder FT-IR spectrum (Fig 1A) showed prominent absorption peaks of chitosan at 896, 1087, 1598, 1653 and 3430 cm^{-1} representing pyranose ring, glucoside, amino, acetamide and hydroxyl groups, respectively [26]. The spectrum of CS-HOBt showed additional peaks at 748 and 1389 cm^{-1} which are attributable to aromatic and azobenzene rings in the HOBt structure. Peaks of phosphate compound in the spectrum of CS-TPP were found at 768 and 1223 cm^{-1}. Strong peaks at 1400 and 1629 cm^{-1}, corresponding to carboxylic acid salt, were observed in CS-EDTA spectrum [27].

NMR spectra are shown in Fig 1B. It shows that the NMR spectrum of CS-acetate showed chemical shifts of the N-acetylglucosamine unit (3.1 ppm) and acetyl group (2 ppm), whereas CS-HOBt showed chemical shifts of aromatic and azobenzene ring at 7.3 and 7.8 ppm. The chemical shifts at 2.6, 5.5 and 8 ppm, corresponding to sulfide group, phosphate group and pyrimidine, respectively, were found in CS-TPP NMR spectrum. Carboxyl chemical shift (3.7 and 4 ppm) was observed in the CS-EDTA NMR spectrum.

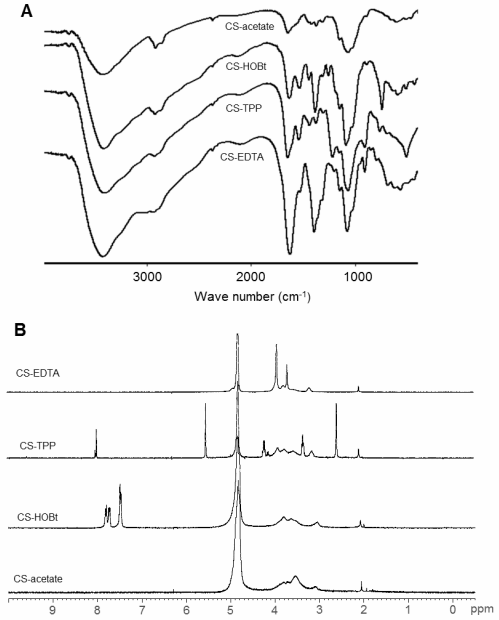

Figure 1: (A) FT-IR and (B) NMR spectra of CS salts

Superoxide radical scavenging

Figure 2 shows the scavenging effects of the CS salt on superoxide radicals. All the salts scavenged superoxide in a concentration-dependent manner. IC_{50} value is the concentration of the sample required to achieve 50 % scavenging of the superoxide free radical and it was determined from the plot of % scavenging against concentration. The IC_{50} of CS-acetate, CS-EDTA, CS-TPP and CS-HOBt was 0.349, 0.611, 0.890 and 1.342 mg/mL, respectively. Thus, the order of

scavenging activity was: CS-acetate > CS-EDTA > CS-TPP > CS-HOBt. The scavenging activity of CS-acetate and CS-EDTA at 2 mg/mL was 87.6 and 76.4 %, respectively.

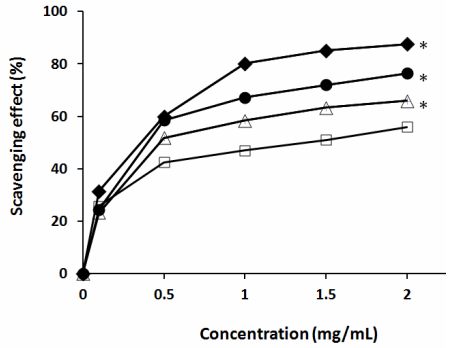

Figure 2: Scavenging activity (n = 3) of CS salts against superoxide radicals: CS-acetate (◆),CS-HOBt (□), CS-TPP (Δ) and CS-EDTA (●);..* indicates significance difference at $p < 0.05$

Hydroxyl radical scavenging activity of chitosan salts

The scavenging activity of the chitosan salts against hydroxyl radicals is shown in Fig 3. The IC_{50} of CS-HOBt, CS-TPP, CS-acetate and CS-EDTA were 0.34, 0.853, 1.43 and 1.54 mg/mL, respectively, giving a scavenging order of CS-HOBt > CS-TPP > CS-acetate > CS-EDTA.

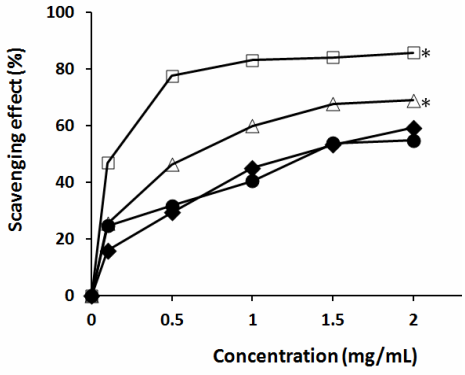

Figure 3: Scavenging activity (n = 3) of CS salts against hydroxyl radicals; CS-acetate (◆),CS-HOBt (□), CS-TPP (Δ) and CS-EDTA (●);* indicates significance difference at $p < 0.05$

Metal ion chelating activity of chitosan salts

The ferrous ion-chelating activity of the CS-salts is shown in Fig 4. The chelating effect of CS-acetate, CS-HOBt and CS-TPP was approximately 20 % at 2 mg/mL. However, CS-EDTA showed the highest chelating effect which was approximately 100 % at 2 mg/mL.

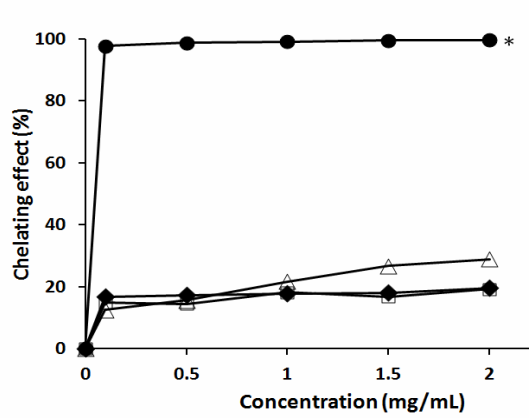

Figure 4: Metal-ion chelating activity (n = 3) of CS salts; CS-acetate (◆), CS-HOBt (□), CS-TPP (Δ) and CS-EDTA (●);* indicates significance difference at $p < 0.05$

Reducing power of chitosan salts

Figure 5 shows the reducing power of the CS salts. The results reveal that the reducing power of CS salts were not-concentration dependent. The reducing power of CS-acetate, CS-HOBt, CS-TPP and CS-EDTA at 1 mg/mL was 0.19, 0.09, 2.40 and 0.08, respectively.

DISCUSSION

Superoxide anion radicals are generated by a number of cellular reactions, including various enzyme systems, such as lipoxygenases, peroxidase, NADPH oxidase and xanthine oxidase. They play an important regulatory role in the formation of other cell-damaging free radicals, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems [28]. The scavenging effect

of ascorbic acid on superoxide radical at 2 mg/mL was 68.2 % [8]. These results indicated that the superoxide radical scavenging activities of CS-acetate and CS-EDTA at the same level were higher than that of ascorbic acid, while those of CS-TPP and CS-HOBt were lower. This might be due to the ability of TPP and HOBt to bond with the hydroxyl and amino groups of CS and hence the hydroxyl and amine groups of CS were not free to react with the superoxide**.**

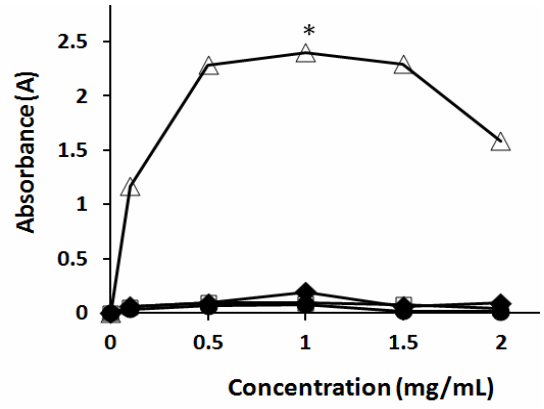

Figure 5: Reducing power (n = 3) of CS salts: CS-acetate (◆), CS-HOBt (□), CS-TPP (Δ) and CS-EDTA (●); * indicates significance difference at $p < 0.05$

Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions such as Cu^{2+} or Fe^{2+}. However, Cu^{2+} and Fe^{2+} do not exist free in body but they can be bound with albumin, ATP, citrate, DNA and membrane lipids [29]. In this study, the hydroxyl radical produced by the reaction of Fe^{2+}-EDTA complex with H_2O_2 in the presence of ascorbic acid, attacks deoxyribose to form products that upon heating with 2-thiobarbituric acid under acidic conditions, yield a pink tint. Added hydroxyl radical scavengers compete with deoxyribose for the resulting hydroxyl radicals and diminish tint formation [30]. Xing et al [8] found, with regard to the scavenging effects on hydroxyl radical, that IC_{50} of chitosan sulfate was in the range of 0.350 – 3.269 mg/mL while the IC_{50} of ascorbic acid was 1.537 mg/mL. In this study, CS-HOBt and CS-TPP showed higher scavenging effect on hydroxyl radical. The hydroxyl radical scavenging mechanism of CS salt to be further investigated.

The results showed that CS-EDTA exhibited the highest metal ion chelating activity of all the CS salts. Xing et al [8] previously reported that the chelating activity of chitosan sulfate was low; and was not greater than 40% at 1 g/mL. These results indicated that CS-acetate, CS-HOBt and CS-TPP had low chelating effect compared with CS-EDTA. EDTA is strongly chelating agent itself.

The results showed that CS-TPP had the highest reducing power. Yen *et al* [11] reported reducing power of approximately 0.2 at 1 mg/mL for crab chitosans while. Zhong *et al* [10] found that reducing power of low molecular weight (MW = 4 kDa) chitosan was approximately 0.06 at 1 mg/mL. Thus, CS-acetate, CS-HOBt and CS-EDTA had reducing power similar to the previous study. The high reducing power of CS-TPP is due to the structure of TPP that can itself act as a reducing agent.

CONCLUSION

The results of the present work indicated that CS salts possess varying levels of antioxidant and free radical scavenging activities, including superoxide and hydroxyl radicals scavenging, metal ion chelating activity and reducing power. CS-acetate showed the highest superoxide radical scavenging effect while CS-HOBt and CS-TPP showed the greatest hydroxyl radical scavenging activities. CS-EDTA and CS-TPP had the highest chelating effect and reducing power, respectively. Overall, the results indicate that the antioxidant activity of chitosan (CS) can be improved by formation of its salts. These

salts may be useful as a source of antioxidants in pharmaceutical products.

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REFERENCES

1. *Kumar MNVR. A review of chitin and chitosan applications. React Funct Polym 2000; 46: 1-27.*
2. *Rinaudo M. Chitin and chitosan: properties and applications. Prog Polym Sci 2006; 31: 603-632.*
3. *Jayakumara R, Menona D, Manzoora K, Naira SV, Tamurab H. Biomedical applications of chitin and chitosan based nanomaterials-A short review. Carbohydr Polym 2010; 82: 227-232.*
4. *Xue C, Yu G, Hirata T, Terao J, Lin H. Antioxidative activities of several marine polysaccharides evaluated in a phosphatidylcholine-iposomal suspension and organic solvents. Biosci Biotech Bioch 1998; 62: 206-209.*
5. *Lin HY, Chou CC. Antioxidative activities of water-soluble disaccharide chitosan derivatives. Food Res Int 2004; 37: 883-889.*
6. *Guo Z, Xing R, Liu S, Yu H, Wang P, Li C, Li P. The synthesis and antioxidant activity of the Schiff bases of chitosan and carboxymethyl chitosan. Bioorg Med Chem Lett 2005; 15: 4600-4603.*
7. *Xing R, Liu S, Yu H, Guo Z, Li Z, Li P. Preparation of high-molecular weight and high-sulfate content chitosans and their potential antioxidant activity in vitro. Carbohydr Polym 2005; 61: 148-154.*
8. *Xing R, Yu H, Liu S, Zhang W, Zhang Q, Li Z, Li P. Antioxidant activity of differently regioselective chitosan sulfates in vitro. Bioorg Med Chem 2005; 13: 1387–1392.*
9. *Guo Z, Liu H, Chen X, Jia X, Lia P. Hydroxyl radicals scavenging activity of N-substituted chitosan and quaternized chitosan. Bioorg Med Chem Lett 2006; 16: 6348-6350.*
10. *Zhong Z, Ji X, Xing R, Liu S, Guo Z, Chen X, Li P. The preparation and antioxidant activity of the sulfanilamide derivatives of chitosan and chitosan sulfates. Bioorg Med Chem 2007; 15: 3775-3782.*
11. *Yen MT, Yang JH, Mau JL. Antioxidant properties of chitosan from crab shells. Carbohydr Polym 2008; 74: 840–844.*
12. *Xing R, Liu S, Guo Z, Yu H, Zhong Z, Ji X, Li P. Relevance of molecular weight of chitosan-N-2-hydroxypropyl trimethyl ammonium chloride and their antioxidant activities. Eur J Med Chem 2008; 43: 336-340.*
13. *Zhong Z, Zhong Z, Xing R, Li P, Mo G. The preparation and antioxidant activity of 2-[phenylhydrazine (or hydrazine)-thiosemicarbazone]-chitosan. Int J Biol Macromol 2010; 47: 93-97.*
14. *Anraku M, Fujii T, Kondo Y, Kojima E, Hata T, Tabuchi N, Tsuchiya D, Goromaru T, Tsutsumi H, Kadowaki D, et al. Antioxidant properties of high molecular weight dietary chitosan in vitro and in vivo. Carbohydr Polym 2011; 83: 501-505.*
15. *Fangkangwanwong J, Akashi M, Kida T, Chirachanchai S. Chitosan-hydroxybenzotriazole aqueous solution: a novel water-based system for chitosan functionalization. Macromol Rapid Comm 2006; 27: 1039-1046.*
16. *Opanasopit P, Techaarpornkul S, Rojanarata T, Ngawhirunpat T, Ruktanonchai U. Nucleic acid delivery with chitosan hydroxybenzotriazole. Oligonucleotides 2010; 20: 127-136.*
17. *Charernsriwilaiwat N, Opanasopit P, Rojanarata T, Ngawhirunpat T, Supaphol P. Preparation and characterization of chitosan-hydroxybenzotriazole/polyvinyl alcohol blend nanofibers by the electrospinning technique. Carbohydr Polym 2010; 81: 675-680.*
18. *Engel PC; Engel PC (Eds). Enzyme cofactors. California: , Enzymology LabFax, Academic; 1996; p 244.*
19. *Rojanarata T, Opanasopit P, Techaarpornkul S, Ngawhirunpat T, Ruktanonchai U. Chitosan-Thiamine Pyrophosphate as a Novel Carrier for siRNA Delivery. Pharm Res 2008; 25: 2807-2814.*
20. *Loretz B, Schnurch AB. In vitro evaluation of chitosan-EDTA conjugate polyplexes as a nanoparticulate gene delivery system. AAPS Journal 2006; 8: E756-E764.*
21. *Cui F, He C, He M, Tang C, Yin L, Qian F, Yin C. Preparation and evaluation of chitosan-ethylenediaminetetraacetic acid hydrogel films for the mucoadhesive transbuccal delivery of insulin. J Biomed Mater Res 2009; 89A: 1063-1071.*
22. *Nishikimi M, Rao NA, Yagi K. The Occurrence of Superoxide Anion in the Reaction of Reduced Phenazine Methosulfate and Molecular Oxygen. Biochem Bioph Res Co 1972; 46: 849-854.*
23. *Halliwell B, Gutteridge JMC, Aruoma OI. The Deoxyribose Method: A Simple "Test-Tube" Assay for Determination of Rate Constants for Reactions of Hydroxyl Radicals. Anal Biochem 1987; 165: 215-219.*
24. *Decker EA, Welch B. Role of Ferritin as a Lipid Oxidation Catalyst in Muscle Food. J Agr Food Chem 1990; 38: 674-677.*
25. *Oyaizu M. Studies on products of browning reaction: Antioxidative activities of products of*

browning reaction prepared from glucosamine. Jpn J Nutri 1986; 44: 307-315.

26. *Fang T, Du Y, Li J, Hu Y, Kennedy JF. Enhancement of antioxidant activity of chitosan by irradiation. Carbohydr Polym 2008; 73: 126-132.*
27. *Günzler, Helmut; Gremlich, Hans–Ulrich editors. IR spectroscopy an introduction. Mörlenbach: Strauss offsetdruck; 2002: 170P.*
28. *Bloknina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: A review. Ann Bot 2003; 91: 179-194.*
29. *Gutteridge JMC. Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. Biochem J 1984; 224: 761-767.*
30. *Cheng Z, Ren J, Li Y, Chang W, Chen Z. Study on the multiple mechanisms underlying the reaction between hydroxyl radical and phenolic compounds by qualitative structure and activity relationship. Bioorg Med Chem 2002; 10: 4067-4073.*