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ARTICLE

Characteristics and Antioxidant Activity of *Elsholtzia splendens* Extract-Loaded Nanoparticles

Ji-Soo Lee,[†] Gun-Hee Kim,[‡] and Hyeon Gyu Lee*,[†]

[†]Department of Food and Nutrition, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791, Republic of Korea, and [‡]Plant Resources Research Institute, Duksung Women's University, 419 Ssangmun-dong, Dobong-gu, Seoul 132-174, Republic of Korea

Elsholtzia splendens extract-loaded chitosan nanoparticles prepared by ionic gelation were characterized by particle size, zeta potential, entrapment efficiency, and loading efficiency. As the initial concentration of *E. splendens* extract was increased, the loading efficiency and zeta potential significantly increased, whereas the entrapment efficiency and particle size significantly decreased. The optimum concentration of *E. splendens* extract for maximum loading efficiency was found to be 0.8 mg/mL. Both free *E. splendens* extract and *E. splendens* extract-loaded chitosan nanoparticles showed concentration-dependent antioxidant activity. However, the lipid peroxidation inhibitory activity of *E. splendens* extract was effectively enhanced when it was entrapped within chitosan nanoparticles. Chitosan nanoparticle encapsulation is therefore a potentially valuable technique for improving the antioxidant activity of *E. splendens* extract.

KEYWORDS: *Elsholtzia splendens*; chitosan nanoparticles; loading efficiency; antioxidant activity; β-carotene bleaching assay

INTRODUCTION

Elsholtzia splendens, a subclass of the family Labiatae, is mainly distributed across eastern Asia (*1*). In folk medicine, especially in Korea and China, *E. splendens* has for a long time been a traditional treatment for a variety of disorders such as coughs, headaches, fevers, diarrhea, and edema (*1*, *2*). Many recent studies have focused on the diverse health-promoting properties of *E. splendens* (*3*−*7*). According to studies regarding the bioactivities of *E. splendens*, the oral administration of *E. splendens* extract inhibited both acute and subchronic inflammation without any significant acute toxicity (*3*) and improved the blood lipid profile (*5*). *E. splendens* was found to activate the antioxidant defense system against 7,12-dimethylbenz(a)anthracene (DMBA)-induced oxidative stress (*6*) and reduce several biomarkers of oxidative stress such as thiobarbituric acid reactive substance, protein carbonyls, and serum 8-hydroxy-2′-deoxyguanosine (*7*). These diverse biological activities of *E. splendens* are known to be induced by flavonoids (*8*). Because of its various biological activities and long history of safe usage (*1*), there has been a recent growing interest in the role of *E. splendens* as an important novel, functional food and pharmaceutical ingredient.

Although the identification of a new natural bioactive material such as *E. splendens* offers an opportunity to develop a new functional food product, its bioavailability needs to be improved as its effectiveness can be decreased by its stability (*9*). Fortunately, the stability of sensitive bioactive materials to an oxidative environment during food processing and storage and from oral administration to absorption can be effectively improved using encapsulation technology (*10*, *11*). Encapsulation has been widely used to protect entrapped substances from adverse environmental conditions including light, oxygen, pH, and digestible enzymes, thus contributing to an increased shelf life and bioavailability of the encapsulated material (*12*). One particular advantage of nanoencapsulation as a delivery vehicle for bioactive materials lies in the fact that water-insoluble substances such as phytosterols, carotenoids, and natural antioxidants have increased apparent solubility, thus increasing their potential applications to the food and pharmaceutical industries (*13*).

Chitosan, a natural biopolymer derived by partial deacetylation of chitin, is a polycationic linear copolymer of β-(1→4)-linked 2-amino-2-deoxy-D-glucan. Chitosan has been widely used as an oral delivery system for many bioactive agents due to its nontoxic, biocompatible, and biodegradable characteristics (*14*). Because chitosan also has a good bioadhesive property, a chitosan delivery system can provide incorporated bioactive agents with a much longer residence time in the gastrointestinal track, thereby resulting in improved bioavailability (*15*, *16*). Recently, there has been growing interest in delivery systems prepared by ionic gelation between the protonated amino groups of chitosan and anions, such as tripolyphosphate (TPP), because of their very simple and mild preparation conditions, homogeneous particle sizes, and bioadhesive properties (*14*). Therefore, it seemed reasonable that a chitosan−TPP nanosystem could be used as an oral delivery carrier for natural bioactive substances such as *E. splendens*.

The goal of the present study is to prepare and investigate the characteristics and antioxidant activity of chitosan-based nanoparticles loaded with *E. splendens* extract. Characteristics of these

*Corresponding author (telephone +82-2-2220-1202; fax +82-2-2281-8285; e-mail hyeonlee@hanyang.ac.kr).

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nanoparticles, including particle size, zeta potential, entrapment efficiency, and loading efficiency, were investigated with different concentrations of the *E. splendens* extract. The flavonoid compounds in *E. splendens* extract were compared before and after loading onto chitosan nanoparticles. The antioxidant activity of *E. splendens* extract-loaded nanoparticles was investigated using the α,α-diphenyl-β-picrylhydrazyl (DPPH) scavenging method and the β-carotene bleaching assay.

MATERIALS AND METHODS

Materials. *E. splendens* was provided by Plant Resources Research Institute, Duksung Women's University. Chitosan with a deacetylation degree of 86.6% and a low molecular weight was purchased from Sigma-Aldrich Chemical Co. (Sigma, St. Louis, MO). Diethylene glycol and β-carotene were also purchased from Sigma-Aldrich Chemical Co. All other chemicals were analytical grade reagents.

Preparation of Chitosan Nanoparticles Loaded with *E. splendens* Extract. The *E. splendens* extract was prepared under optimal extraction conditions determined by a preliminary study. Briefly, ground, dried *E. splendens* flowers were mixed at 1% (w/v) with 54.6% ethanol, and extraction was carried out under magnetic stirring at 400 rpm for 116.8 min at room temperature. Upon completion of extraction, the extracts were filtered through Whatman no. 41 filter paper (Whatman Inc., Clifton, NJ) and were vacuum-dried overnight in a centrifugal vacuum concentrator (Biotron Inc., Puchon, Korea).

Chitosan nanoparticles loaded with *E. splendens* extract were prepared according to the procedure described by Jang and Lee (*17*). Chitosan was dissolved in 1% acetic acid, and *E. splendens* extract was slowly added to give a final concentration ranging from 0.0 to 1.0 mg/mL. Under magnetic stirring (1000 rpm) at room temperature, 3 mL of 1.6 mg/mL TPP solution was added into 5 mL of chitosan solution containing *E. splendens* extract using a cassette tube pump (EYELA SMP-21, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at a flow rate of 1 min/mL, so that the final concentrations of chitosan and TPP were 1.5 and 0.6 mg/mL, respectively.

Physiochemical Properties of Chitosan Nanoparticles Loaded with *E. splendens* Extract. The particle size and zeta potential of *E. splendens* extract-loaded chitosan nanoparticles were determined by quasielastic laser light scattering with a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, U.K.). All measurements were performed in triplicate.

Determination of Entrapment and Loading Efficiencies. The entrapment efficiency (EE) and loading efficiency (LE) of *E. splendens* extract-loaded nanoparticles were indirectly determined by measuring the total flavonoid amount entrapped within the nanoparticles (*17*, *18*). The amount of total flavonoid entrapped within the nanoparticles was determined by measuring the amount of free total flavonoid in the supernatant recovered after ultracentrifugation (Optima TL ultracentrifuge, Beckman, Fullerton, CA) at 15000*g* for 30 min, using the method of Abeysinghe et al. (*19*) with some modification. To 0.14 mL of extract were added and mixed 0.7 mL of diethylene glycol and 0.07 mL of 1 N sodium hydroxide. The contents of the tubes were mixed and incubated for 1 h at 37 °C, and then the absorbance was measured at 420 nm using a spectrophotometer (DU 650, Beckman Coulter Inc., Fullerton, CA).

The EE and LE of the *E. splendens* extract-loaded nanoparticles were calculated using eqs **1** and **2**:

$$\text{entrapment efficiency (\%)} = \frac{\text{actual amount of } \textit{E. splendens} \text{ extract entrapped in nanoparticles}}{\text{theoretical amount of } \textit{E. splendens} \text{ extract entrapped in nanoparticles}} \times 100 \quad (1)$$

$$\text{loading efficiency (\%)} = \frac{\text{actual amount of } \textit{E. splendens} \text{ extract entrapped in nanoparticles}}{\text{weight of nanoparticles}} \times 100 \quad (2)$$

Analysis of Flavonoid Compounds. The analysis of flavonoid compounds in *E. splendens* extract before and after nanoencapsulation was performed using high-performance liquid chromatography (HPLC). Both samples, the free *E. splendens* extract before nanoencapsulation and the *E. splendens* extract-loaded nanoparticles, were individually mixed with 0.2% butylated hydroxyanisole (BHA) in 62.5% methanol, and then 6 M HCl was carefully added to yield the final concentration of 1.2 M HCl and 50% methanol. Each mixture was refluxed for 2 h in a 90 °C water bath and then allowed to cool in the refrigerator (*20*). A 20 μL sample was injected into the HPLC after filtration through a 0.45 μm filter. HPLC separation was performed using a Dionex ultimate 3000 series (Dionex, Sunnyvale, CA) with a Luna C_{18} column (4.6 × 250 mm, 5 μm, Phenomenex, Torrance, CA). The mobile phase consisted of 0.1% formic acid in 30% methanol solution (A) and 100% methanol (B). Sample was eluted a flow rate of 0.5 mL/min with a linear gradient of 25−68% B in A over 35 min. The flavonoid compounds were identified according to the retention times of the authentic standards (hesperidin, rutin, quercetin, kaempferol, and apigenin) monitored by UV detection at 260 nm

Determination of Antioxidant Activity. *α,α-Diphenyl-β-picryl-hydrazyl (DPPH) Radical Scavenging Activity*. The scavenging effect of the *E. splendens* extract-loaded nanparticles on DPPH free radicals was measured according to the method of Brand-Williams et al. (*21*) with minor modification (*22*). DPPH (16 mg) was dissolved in 100 mL of ethanol, and then 100 mL of distilled water was added. This 10-fold diluted extract (200 μL) was then mixed with 0.8 mL of DPPH solution to initiate the reaction. The mixture was shaken and left for 30 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer. The DPPH radical scavenging effect was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100 \quad (3)$$

β-Carotene Bleaching Method. The effect of the *E. splendens* extract-loaded nanoparticles on the lipid peroxidation was determined according to the β-carotene bleaching method following modification of the procedure described by Yang et al. (*23*). In round-bottom flasks containing 0.02 mL of linoleic acid and 0.2 mL of Tween 20, 0.2 mg/mL of β-carotene solution and 1 mL of chloroform were added. After evaporation to dryness under vacuum at 50 °C for 5 min, 150 mL of oxygenated distilled water was added, and the mixture was mixed with a magnetic stir bar to form an emulsion. A 15 mL aliquot of the emulsion was added to a tube containing free *E. splendens* extract or *E. splendens* extract-loaded nanoparticles at concentrations ranging from 0.02 to 0.25 mg *E. splendens* extract per 1 mL of emulsion. The samples were then subjected to thermal autoxidation at 50 °C for 2 h. The absorbance of the solution at 470 nm was monitored by spectrophotometer measurements taken at 30 min intervals. All samples were assayed in triplicate. Antioxidant activity was calculated as percent inhibition relative to the control using the equation

$$\text{antioxidant activity} = \left(1 - \frac{A_0 - A_t}{A0_0 - A0_t}\right) \times 100 \quad (4)$$

where A_0 is the initial absorbance of the sample, A_t is the absorbance of the sample after 120 min, $A0_0$ is the initial absorbance of the control, and $A0_t$ is the absorbance of the control after 120 min.

Statistical Analysis. All experiments were performed in triplicate. All data were expressed as mean value ± standard deviation. The significance of differences ($p \leq 0.05$) among the corresponding mean values was determined using one-way analysis of variance (ANOVA) followed by Duncan's multiple-comparison test (SAS 9.1, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Physicochemical Characterization of *E. splendens* Extract-Loaded Nanoparticles. Characteristics of the nanoparticles, including particle size and zeta potential, were investigated with different concentrations of *E. splendens* extract. **Figure 1** shows the influence of *E. splendens* extract concentration on the particle size and zeta potentials of the nanoparticles. The presence of *E. splendens* extract within chitosan particles significantly increased

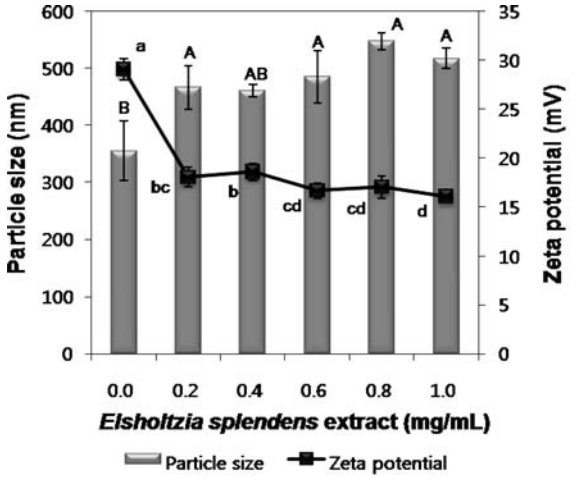

Figure 1. Particle sizes and zeta potentials of chitosan nanoparticles with different concentrations of *E. splendens* extract. Different letters (A−D and a−f) indicate a significant difference in entrapment efficiency (EE) or loading efficiency (LE), respectively.

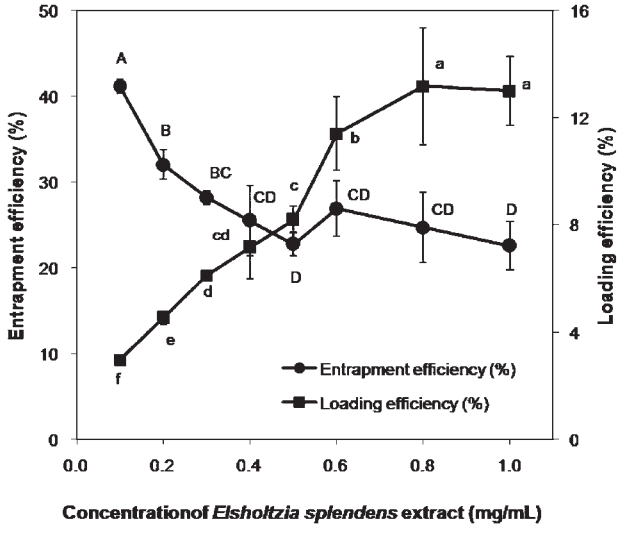

Figure 2. Entrapment and loading efficiencies of chitosan nanoparticles with different concentrations of *E. splendens* extract. Different letters (A−B and a−d) indicate a significant difference in particle size or zeta potential, respectively.

the particle size. As the *E. splendens* extract concentration was increased from 0.2 to 1.0 mg/mL, the particle size of the chitosan particles slightly increased, but not significantly, whereas the LE of chitosan nanoparticles clearly significantly increased (**Figure 2**). These results demonstrated that as the *E. splendens* extract concentration increased, the chitosan nanoparticles with the similar size entrapped more *E. splendens* extract, which suggests that the structure of chitosan nanoparticles becomes denser with less void volume.

The zeta potential of a nanoparticle is its electrical potential and is commonly used to express the stability of nanoparticles in suspension through electrostatic repulsion between the particles (*24*, *25*). As the *E. splendens* extract concentration was increased from 0.0 to 1.0 mg/mL, the zeta potential of the chitosan nanoparticles significantly decreased from approximately 30 to 15 mV. Similar results have been reported by Kim et al. (*26*) and Gan and Wang (*24*) for retinol- and bovine serum albumin-entrapped chitosan nanoparticles, respectively. A decrease of zeta potential indicates a decrease of the surface charges on the particles, forcing less repulsive forces between the particles and creating a higher tendency to aggregate.

The zeta potential of nanoparticles can be influenced by the surface charge of particles and the particle size, because the value of the zeta potential is determined by the electrophoretic mobility of the particle. The decrease of zeta potential in the present study could be because the increase of particle size induced by an increase of *E. splendens* extract concentration has retarded the electrophoretic mobility of the particle.

Effect of *E. splendens* Extract Concentration on the EE and LE of the Nanoparticles. The EE and LE of the *E. splendens* extract-loaded chitosan nanoparticles were determined by comparing the amount of bioactive flavonoid substance in the *E. splendens* extract before and after nanoencapsulation. **Figure 2** shows the effect of *E. splendens* extract concentration on the EE and LE of chitosan nanoparticles. The EE significantly decreased from approximately 41 to 23% as the initially loaded concentration of *E. splendens* extract increased from 0.1 to 1.0 mg/mL. This result could be due to a higher concentration of *E. splendens* extract causing a decrease in the chitosan available to entrap the *E. splendens* extract. A similar phenomenon has also been observed for chitosan nanoparticles containing catechin, a bioactive polyphenol in green tea (*27*). The initial concentration of *E. splendens* extract also significantly influenced the LE of nanoparticles. LE significantly increased as the *E. splendens* extract concentration increased up to 0.8 mg/mL and then slightly decreased, but not significantly, with a further increase in concentration from 0.8 to 1.0 mg/mL. This result could indicate that the concentration ranges above 0.8 mg/mL exceed the acceptable limit within the chitosan nanoparticles. The optimum concentration of *E. splendens* extract for maximum loading efficiency was found to be 0.8 mg/mL.

Flavonoid Compounds before and after Nanoencapsulation. To evaluate the effect of nanoencapsulation on the flavonoid compounds in *E. splendens* extract, the flavonoid compounds were analyzed using HPLC. Typical chromatograms obtained in the analysis of the *E. splendens* extract before and after nanoencapsulation are shown in panels **A** and **B**, respectively, of **Figure 3**. The peaks were identified using the retention times of standards. Four flavonoid compounds were found in the free *E. splendens* extract: hesperidin, apigenin, quercetin, and rutin. This is consistent with the report by Um and Kim (*28*). **Figure 3B** shows that the flavonoids in the *E. splendens* extract-loaded nanoparticles were hesperidine, apigenin, and quercetin. Although rutin, the smallest compound among the four flavonoids, was not found after nanoencapsulation, the other three flavonoid compounds remained after the nanoencapsulation procedure. The nanoencapsulation in this study used reversible physical cross-linking by electrostatic interaction and, unlike other nanoparticulations, such as spraying-drying and reverse micelle−solvent evaporation methods, did not use a heating process or toxic organic solvents; therefore, it might be a suitable oral delivery system for sensitive bioactive materials, such as flavonoids.

Antioxidant Activity of *E. splendens* Extract-Loaded Nanoparticles. The in vitro antioxidant activities of *E. splendens* extract-loaded chitosan nanoparticles prepared under conditions of 0.8 mg/mL *E. splendens* extract, 1.5 mg/mL chitosan, and 0.6 mg/mL TPP were investigated using the DPPH method and β-carotene bleaching assay and compared with those of free *E. splendens* extract and *E. splendens* extract-free blank chitosan nanoparticles. DPPH radical scavenging activities of nonentrapped (free) *E. splendens* extract and *E. splendens*-loaded chitosan nanoparticles were compared (**Figure 4**). Blank chitosan nanoparticles did not show any antioxidant activity against

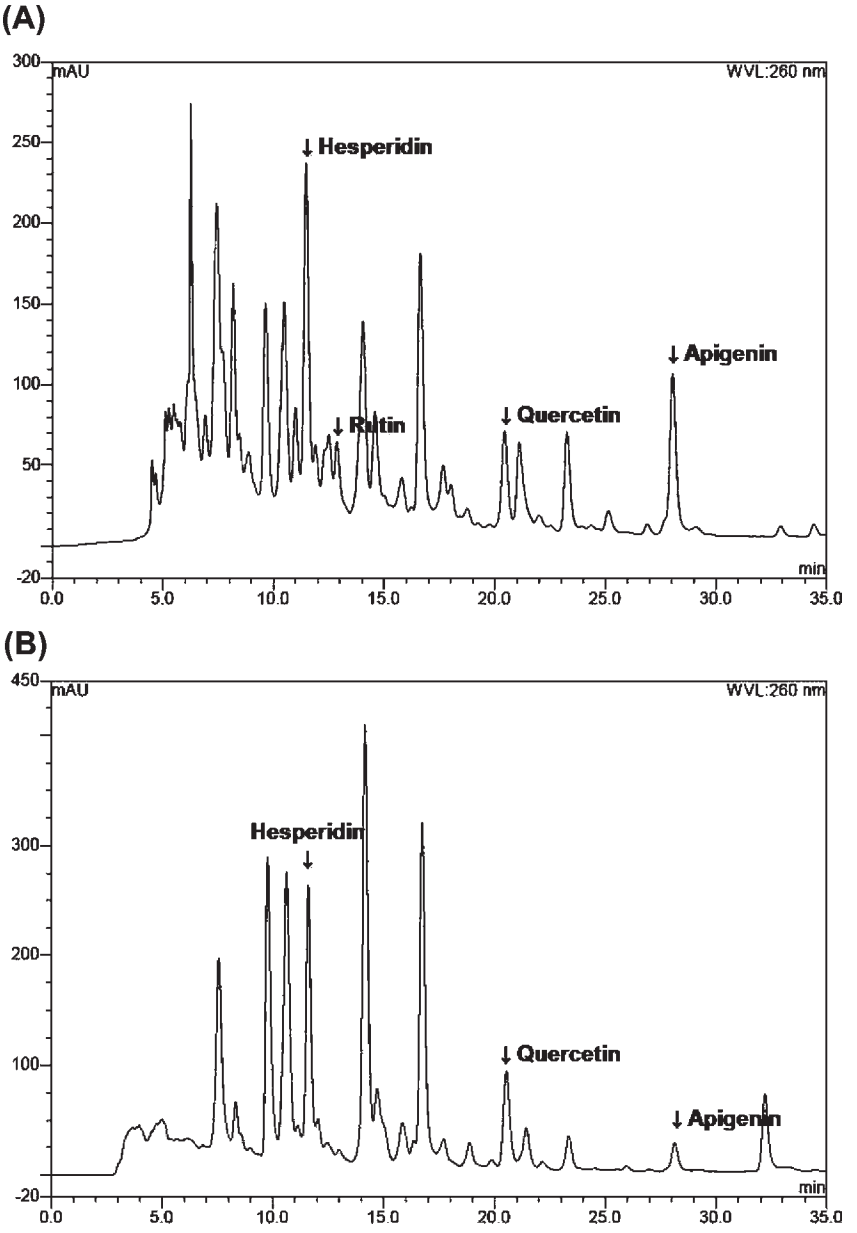

Figure 3. HPLC chromatograms of free *E. splendens* extract (**A**) and *E. splendens* extract-loaded chitosan nanoparticles (**B**).

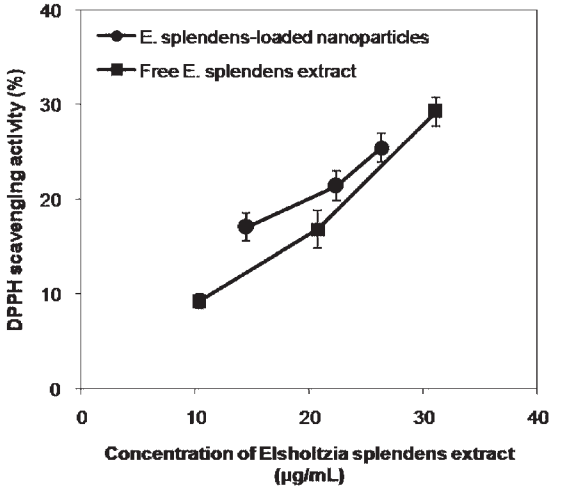

Figure 4. DPPH scavenging activities of free *E. splendens* extract and *E. splendens* extract-loaded chitosan nanoparticles.

DPPH scavenging activity (data not shown). Both free *E. splendens* extract and *E. splendens* extract-loaded nanoparticles showed a concentration-dependent DPPH radical scavenging activity. There was no clear difference in DPPH radical scavenging activities resulting from nanoencapsulation. It can be therefore concluded that the chitosan nanoparticles maintained the *E. splendens* antioxidant activity (*22*).

The effect of *E. splendens* extract-loaded chitosan nanoparticles on lipid peroxidation was investigated using a β-carotene bleaching assay and was compared with the activities of nonentrapped (free) *E. splendens* extract and *E. splendens*-loaded chitosan nanoparticles (**Figures 5** and **6**). **Figure 5** shows the decrease in the absorbance of β-carotene in the presence of free *E. splendens* extract and *E. splendens* extract-loaded chitosan nanoparticles during the 2 h incubation period. During the determination time, the inhibition of lipid peroxidation by free *E. splendens* extract increased with the concentration range from 0.05 to 0.25 mg/mL, which reflects the concentration-dependent antioxidant activity of *E. splendens* ethanol extract.

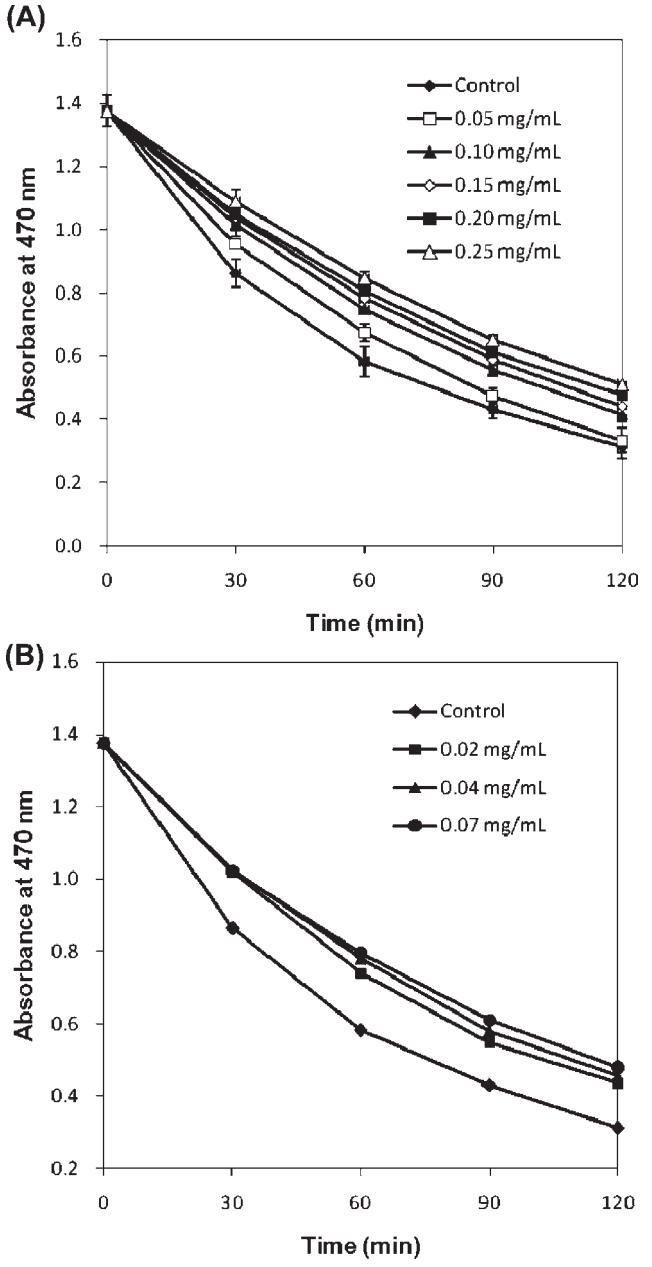

Figure 5. Changes of absorbance at 470 nm with time for free *E. splendens* extract (**A**) and *E. splendens* extract-loaded chitosan nanoparticles (**B**) in β-carotene/linoleic acid emulsion.

The *E. splendens* extract-loaded chitosan nanoparticles also showed a concentration-dependent antioxidant activity in the concentration range from 0.13 to 0.53 mg of nanoparticles/mL (**Figure 5B**), which contained an *E. splendens* extract of 0.02–0.07 mg/mL. However, the *E. splendens*-free blank chitosan nanoparticles did not affect lipid peroxidation (data not shown). **Figure 6** shows the antioxidant activity calculated inhibition of lipid peroxidation by free *E. splendens* extract and entrapped *E. splendens* extract within chitosan nanoparticles after the 2 h incubation. The antioxidant activity of nonentrapped free *E. splendens* extract increased from approximately 2 to 19% as the *E. splendens* extract concentration ranged from 0.05 to 0.25 mg/mL; however, chitosan nanoparticles containing *E. splendens* extract concentrations ranging from only 0.02 to 0.07 mg/mL showed antioxidant activity ranging from 12 to 16% (**Figure 6**). Using the linear equation derived from the antioxidant activity of free and entrapped *E. splendens* extracts, achievement of a 15% inhibition

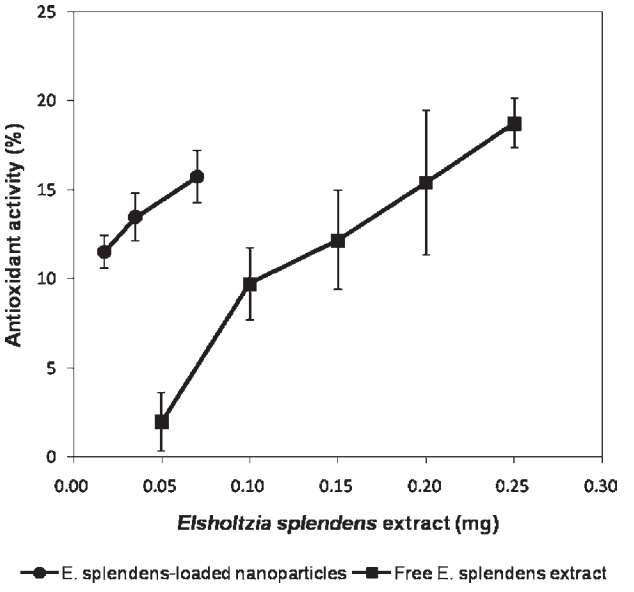

Figure 6. Antioxidant activities of free *E. splendens* extract and *E. splendens* extract-loaded chitosan nanoparticles.

of the lipid peroxidation required concentrations of 0.19 and 0.06 mg/mL on the free and entrapped *E. splendens* extract, respectively. The improved antioxidant activity of *E. splendens* extract by nanoencapsulation could have been due to the increased stability of the incorporated extract bioactive substances such as flavonoids in chitosan nanoparticles and the improved solubility of *E. splendens* extract in the aqueous system. A protective effect of encapsulation on incorporated sensitive substances, such as catechin, α-tocopherol, oryzanol, and vitamin C has been reported (*10*, *17*, *29*−*31*). In addition, the water solubility of poorly soluble substances can be enhanced by a reduction in particle size because the diminished size leads to increased surface area, saturation solubility, and dissolution velocity (*32*). Enhanced water solubility was shown to produce increased in vitro availability in aqueous systems and in vivo bioavailability (*33*, *34*). These results clearly demonstrate that the antioxidant activity of *E. splendens* extract can be effectively improved by chitosan nanoencapsulation.

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