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Release characteristics of vitamin E incorporated chitosan microspheres and *in vitro–in vivo* evaluation for topical application

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ABSTRACT

Vitamin E (VE) is known to be a natural antioxidant by scavenging free radicals and leading to an antiaging effect. Chitosan (CS), an excellent biopolymer obtainable from renewable resources has become of great interest as a new functional material of high potential in various fields. The use of VE in cosmetic products is limited due to its low stability. In this study, aiming to solve the stability problem, VE was successfully incorporated into CS microspheres. Characterization of the particles and *in vitro* release studies were performed in detail. *In vivo* analyses results showed that the VE formulation prepared has enhanced the skin moisture and skin elasticity while decreasing the skin wrinkle volume which means CS microspheres containing VE is a promising candidate as an antiaging product.

1. Introduction

Encapsulation technique for lipophilic active ingredients is used particularly in the fields of cosmetology and dermatology. Chitosan (CS) has become of great interest as a new functional material of high potential in various fields (Kumar, 2000). CS is a biopolymer obtainable from renewable resources such as crustacean shells (Hudson & Smith, 1998). CS is the only polycationic polysaccharide in nature with promising biocompatibility, biodegradability and low toxicity. Antibacterial characteristics in biomedicine, hydrating properties in cosmetics and other beneficial features of CS especially in emulsions and cosmetic gels were reported (Caiqin et al., 2006; He, Guo, & Zhang, 2008).

Vitamin E (VE) is known to be a natural antioxidant by scavenging free radicals and leading to an antiaging effect. It is the most important chain-breaking radical scavenger in the liposoluble compartment, thus constituting the major specific defence line against lipid peroxidation (Papas, 1999; Rozman, Gasperlin, Tinois-Tessoneaud, Pirot, & Falson, 2009). As the outermost organ of the body, skin is frequently and directly exposed to a prooxidative environment including ultraviolet radiation, drugs and air pollutants (Thiele, Dreher, & Packer, 2000).

Dermal delivery by topical preparations such as creams, gels and lotions is limited due to barrier properties of *Stratum corneum*. This limitation hinders the drug deposition and leads to relative poor stability of vitamins by direct exposure to UV light (Roberta, Sonia, Rita, Lorena, & Nevio, 2009).

The use of VE in cosmetic products is limited due to its low stability. Aiming to solve the stability problem, it was incorporated into CS microsphere formulations in this study. Characterization studies, stability analyses, *in vitro* release characteristics and *in vivo* efficacy studies were performed on the microspheres prepared. The major purpose of this work was to formulate a stable cosmetic formulation for VE aiming an enhanced antiaging effect with the increased VE flux through the skin by the help of superior characteristics of an excellent biopolymeric microsphere.

2. Experimental

2.1. Materials

α-Tocopherol (vitamin E) was a kind gift from Roche, Türkiye. CS was purchased from Fluka Chemicals, Germany. Acetic acid, ethanol, sodium chloride, methanol and acetonitrile were purchased from Merck, Germany. All other chemicals and reagents used were of pharmaceutical and analytical grade.

2.2. Methods

2.2.1. Preparation of CS microspheres

Formulation was prepared using spray-drying method (Learoyd, Burrows, French, & Seville, 2009). Briefly, accurately weighed CS (2 g) was dissolved in acetic acid solution (240 ml; 2%, v/v). VE (200 mg) mixed with ethanol (240 ml; 96%) was added to the

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Table 1
Particle size, polydispersity index and zeta potential values of F_1 and F_2.

Particle size (μm) ± SE		Polydispersity index ± SE		Zeta potential (mV) ± SE	
F_1	F_2	F_1	F_2	F_1	F_2
3.482 ± 0.200	0.417 ± 0.113	0.61 ± 0.01	0.97 ± 0.01	44.5 ± 0.0	11.0 ± 0.0

SE: standard error, $n = 3$.

acidic CS solution under mild agitation. Final transparent solution was then spray-dried using mini spray-dryer (Büchi-190, Switzerland) with an inlet temperature of 145 ± 1 °C (outlet temperature 50 ± 1 °C). A white dry powder (F_1) was obtained and kept at room temperature in colored vials until being analyzed. Among the many formulations prepared during preformulation studies, F_1 (VE-polymer ratio, 1:10) was selected for further studies considering the smaller particle size and higher zeta potential values (Table 1). Empty microspheres (F_2) were prepared as described above without the addition of VE. In order to evaluate the *in vivo* antiaging efficacy of the microspheres, CS microspheres containing VE (10%, w/w) were incorporated into a basic cold cream (USP XXI) formulation and applied to human volunteers.

2.2.2. Characterization of microspheres

The morphological structures of CS microspheres were analyzed using the transmission electron microscope (TEM) (FEI Company-Technai™ G^2 Spirit/Bigtum, USA).

The average particle size, size distribution and zeta potential values of formulations prepared were analyzed using Zetasizer Nanoseries (Malvern Instruments, England). Prior to analyses, samples of microspheres were diluted with bidistilled water and adjusted to a constant conductivity of 50 μS cm^{-1} with 0.9% NaCl. Measurements were repeated in triplicate.

Differential scanning calorimeter (DSC) (DSC-60, Shimadzu, Japan), X-ray diffractometer (XRD) (Rikagu, Japan), Fourier transform infrared spectrometer (FT-IR) (Perkin-Elmer, England) and CP-mass NMR (^{1}H NMR) spectrophotometer (Ultra Shield, Germany) were used for the evaluation of the polymeric lattice structure. In this study, the incorporation of VE into CS microspheres was analyzed with the comparison of the spectra of F_1 with F_2 and reference pure CS. For further evaluation of the polymeric structures of CS microspheres, FT-IR analyses were carried out on both formulations prepared. Pure CS was also analyzed and the spectrum was used as a reference.

For the quantification of VE incorporated into CS microspheres, accurately weighed (5 mg) formulation was dissolved in 2% (v/v) acetic acid solution and ethanol (5 ml, 4:1) mixture and agitated at 4000 rpm for 3 min. 1 ml of supernatant was collected and analyzed using a validated HPLC method.

The amount of VE in aliquots was analyzed with HPLC apparatus consisting of a pump (LC 10-AD), a UV detector (SPD-20A), a data station (Shimadzu, Japan) and C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size). The mobile phase was composed of acetonitrile:methanol (95:5, v/v) and was degassed prior to the analyses. Flow rate of the mobile phase was 1.5 ml min^{-1} and 20 μL constant amount of samples were injected via an automatic injector (Shimadzu, Japan) for the analyses. Photodiode array detector (Shimadzu, Japan) was used at 292 nm and the column temperature was set to 30 ± 1 °C.

For the reliability of the data, validation studies of the HPLC method were performed.

Standard mixtures were prepared at three different concentrations of VE and analyzed 6 times a day and at 3 consecutive days to assess the intra-assay precision, intermediate precision and accuracy. All stock solutions were prepared by ethanol and stored at −20 °C until being used. The statistical investigation of the linearity was assessed using linear regression method through r^2 value.

2.2.3. In vitro studies

2.2.3.1. In vitro release studies of VE from CS microspheres. *In vitro* release studies were performed using Franz diffusion cells (Casagrande et al., 2006; Netzlaff, Lehr, Wertz, & Schaefer, 2005). The diffusion cells were thermoregulated with a water jacket at 32.0 ± 0.5 °C. Polypropylene membrane was mounted on a Franz diffusion cell after keeping it in the donor compartment for 20 min. The receptor chamber was filled with ethanol and 1000 μL of the formulation was applied to the donor compartment. 0.5 ml aliquots were withdrawn from the receptor compartment at predetermined time intervals. This amount was replaced by the fresh receptor solution. Aliquots were stored at 4.0 ± 0.5 °C until being analyzed.

2.2.3.2. In vitro antioxidant effect. Antioxidant effect of the formulation was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) test (Lee et al., 2000). In order to compare the antioxidant activity, empty formulations were also tested. For better evaluation of the production temperature (inlet 145 ± 1 °C) effect on the antioxidant activity of VE, DPPH test was performed on pure VE which was heated up to 145 ± 1 °C and cooled down to room temperature (H-VE) (Table 2).

Briefly, 5 ml of each formulation was diluted with ethanol to 25 ml. However, pure VE and H-VE was diluted to 25 ml using methanol instead. Various concentrations of solutions were mixed with the methanolic solution containing DPPH radicals, shaken vigorously and left to stand for 30 min in the dark. All samples were tested in triplicate.

The reduction in DPPH radical was measured by an UV spectrophotometer (Shimadzu UV–Visible Recording Spectrophotometer UV-160 A, Japan) at 517 nm. The radical scavenging activity was calculated as the percentage of DPPH discoloration using Eq. (1). Stable amounts were also determined through extrapolation from linear regression.

$$\text{Scavenging}\,\% = \frac{A_c - A_s}{A_c} \times 100 \quad (1)$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample.

2.2.4. In vivo efficacy tests

In vivo efficacy studies were carried out to determine the constructive influence of the formulation on skin aging. Volunteers used in efficacy tests were not informed about the content of the formulation prior to the study. Formulation was applied twice a

Table 2
Scavenging activity of pure VE, H-VE, F_1 and F_2 according to DPPH test.

Samples	Scavenging % DPPH			
	Pure VE ± SE	H-VE ± SE	F_1 ± SE	F_2 ± SE
250 μL 3 mL^{-1}	98.10 ± 0.00	94.12 ± 0.00	17.63 ± 0.00	0.00
100 μL 3 mL^{-1}	91.89 ± 0.00	93.78 ± 0.00	11.56 ± 0.00	0.00
50 μL 3 mL^{-1}	90.73 ± 0.00	91.85 ± 0.00	5.38 ± 0.00	0.00

SE: standard error, $n = 3$.

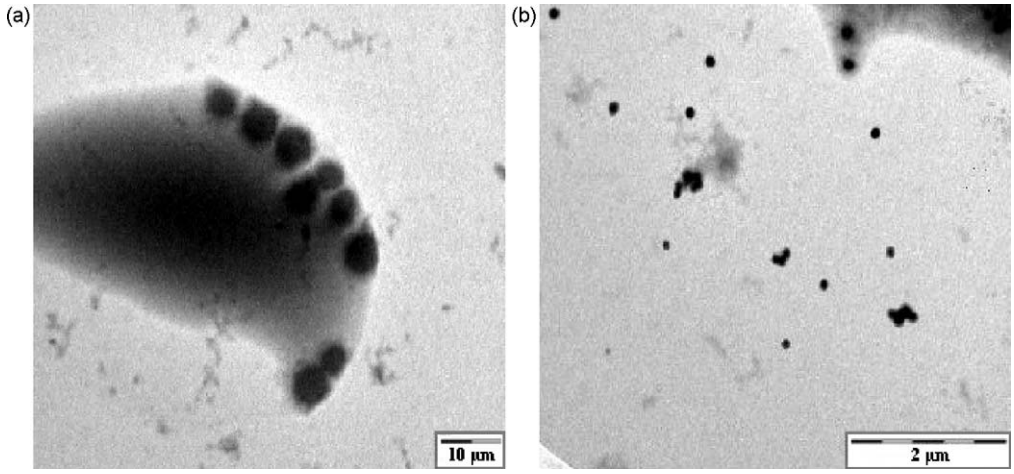

Fig. 1. TEM images of formulations prepared: (a) F_1 and (b) F_2.

day on ten female volunteers (cheeks and crows' feet area) for 1 month and skin measurements were repeated once a week. During the *in vivo* efficacy tests, skin moisture and skin sebum were measured with Corneometer® (Courage & Khazaka, Germany), skin pH with pH-meter® and skin elasticity with Cutometer®. Skin roughness was examined using Skin Visiometer® and VisioScan® (Stäb, Sauermann, & Hoppe, 1997; Yazan, 2006). Statistical evaluation was performed using the two-way-ANOVA test.

The *in vivo* study protocol was approved by the Scientific Ethical Committee of Eskişehir Osmangazi University, Türkiye (Protocol No. 06-08-22-1).

3. Results and discussion

3.1. Characterization of microspheres

Morphological studies showed that particles were round in shape for both of the formulations (Fig. 1). Particle sizes were found to increase ($p < 0.05$) by incorporation of VE; however, F_1 formulation showed relatively homogeneous size distribution considering the polydispersity index data (Table 1) (Başaran, Demirel, Sırmagül, & Yazan, 2010; Cevher et al., 2006; Lopedota et al., 2009). Since the principal aggregation responses are attributed to zeta potential changes, high cationic character of the particles allow the predictions of the stability of the formulations prepared. Incorporation of VE enhanced the cationic character of the particles which is reported to be a triggering factor for the enhancement of topical penetration (Table 1) (Liu et al., 2007). DSC analyses demonstrated no endothermic peaks related to VE incorporation into CS microspheres. This revealed that the polymeric lattice was in amorphous state and there was no interaction between VE and the polymer (Fig. 2). It can be concluded that the formulation process did not change the characteristics of the drug incorporated (Cevher et al., 2006).

DSC and XRD play a prominent role in the characterization of polymeric particles because they are able to provide structural information on the dispersed particles (Bunjes and Unruh, 2007). Therefore in this study for better evaluation of the crystalline structure of polymeric particles DSC and XRD analyses were carried out simultaneously.

XRD analysis results support the DSC (Fig. 3) data showing that pure CS was in the amorphous form prior to the formation of particles. After spray-drying method, no crystalline peaks were seen in the spectra indicating that microspheres were reformed into amorphous state. Incorporation of VE also did not affect the amorphous structure of the particles.

In the FT-IR spectrum, specific peaks of CS were detected at 1630 cm^{-1} (amide) and 1590 cm^{-1} (amine) showing that the production parameters did not affect the structure of the carrier (Shi et al., 2009) (Fig. 4). Typical peaks of VE, 1600–1700 cm^{-1} bands for C=C formation and 900–1200 cm^{-1} for C–O formation were detected in the spectrum of F_1 showing that VE was successfully incorporated into CS microspheres.

^{1}H NMR analysis was applied effectively to characterize the form of VE within the solid matrix, molecular mobility and molecular interactions between drug and excipients (Jenning, Mäder, & Gohla, 2000; Li, Wong, Shuhendler, Rauth, & Yu Wu, 2008). Analyses results showed that F_2 formulation showed almost identical spectrum with pure CS. However, characteristic peaks of VE were seen in the spectrum of F_1 supporting the data obtained from ^{1}H NMR analyses (Fig. 5). It can be concluded that VE was successfully incor-

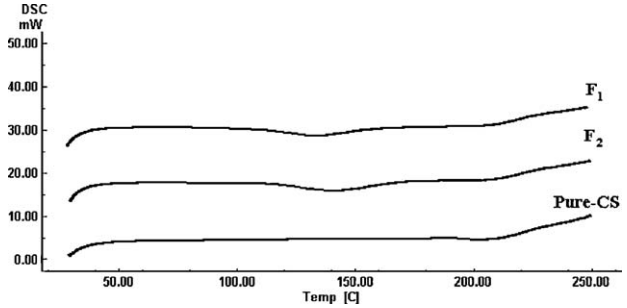

Fig. 2. DSC thermograms of F_1, F_2 and pure CS.

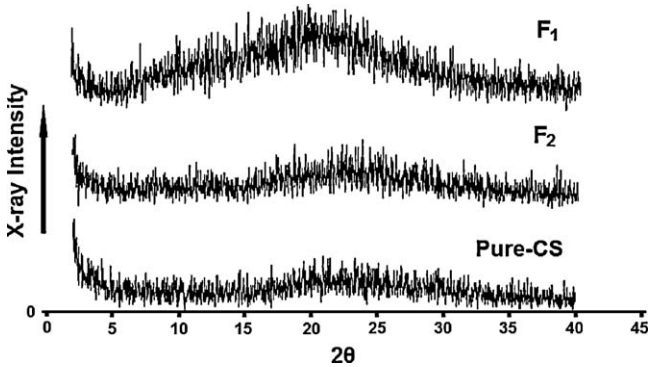

Fig. 3. XRD spectra of F_1, F_2 and pure CS.

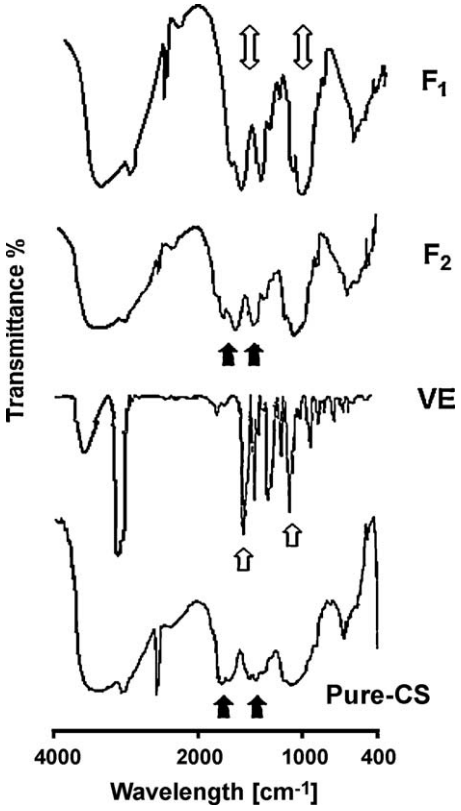

Fig. 4. FT-IR spectra of F_1, F_2, VE and pure CS.

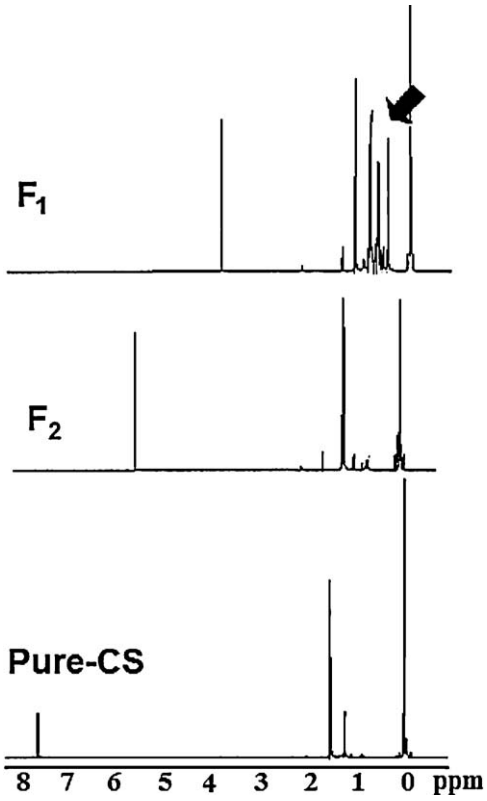

Fig. 5. ^{1}H NMR spectra of F_1, F_2 and pure CS.

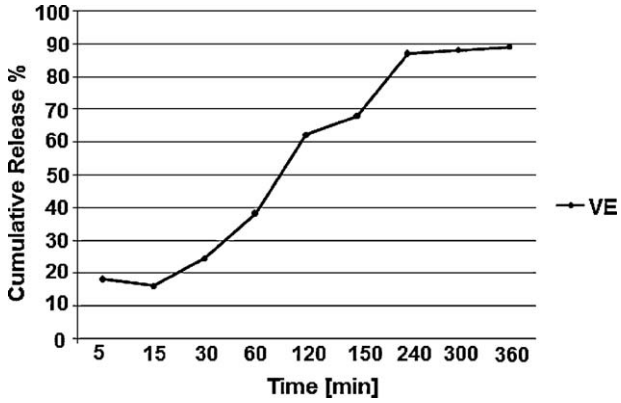

Fig. 6. Release profile of VE from F_1 [mean ± SE, $n = 3$].

porated into CS microspheres (Fernandez-Megia, Novoa-Carballal, Quinoa, & Riguera, 2005).

For the HPLC method developed for quantification of VE linearity in ethanol was found to be $y = 3730.712x - 6796.333$ where $r^2 = 0.999$. Limit of detection (LOD) was determined to be 0.008 μg ml^{-1} while limit of quantitation (LOQ) was 0.025 μg ml^{-1}. The relative retention time of VE was 15 min and this time did not change after spray-drying of formulation. This data verifies the antioxidant effect of vitamin E which did not change after spray-drying.

HPLC analyses results showed that the incorporation efficiency of VE into the microspheres was 78.40 ± 2.41% (w/w).

3.2. In vitro studies

3.2.1. In vitro release studies of VE from the CS microsphere formulation

Franz diffusion cell analyses results showed that the release of VE from the CS microspheres was 89.1% at the end of 6 h (Fig. 6). There was a burst release at the 5th minute which was attributed to VE existing on the surface of the CS microspheres. This portion most probably dissolves initially in the oily phase during the incorporation of the microspheres into the base cold cream. However, the release lasted for 6 h.

3.2.1.1. Franz cell studies. The steady-state flux (J_s) and the permeability coefficient (k_p) of VE from the formulation was calculated to be $2.22 \times 10^{-1} \pm 0.0001$ μg cm^{-2} h^{-1} and $5.358 \times 10^{-4} \pm 0.001$ cm h^{-1}, respectively. The results indicated that the release of VE from the CS microspheres varied depending on time.

3.2.2. In vitro antioxidant effect

DPPH analyses results demonstrated that F_2 formulation showed no antioxidant effect whereas F_1 showed *in vitro* antioxidant effect as expected (Table 2).

3.3. In vivo efficacy tests

As a result of the efficacy studies, a significant increase in skin moisture ($p \leq 0.001$) and elasticity ($p < 0.05$) was determined. There was a significant decrease in roughness ($p \leq 0.01$) while no changes occurred in skin pH and sebum values ($p > 0.05$) (Fig. 7). Therefore, the formulation prepared in this study is a good candidate for skin antiaging.

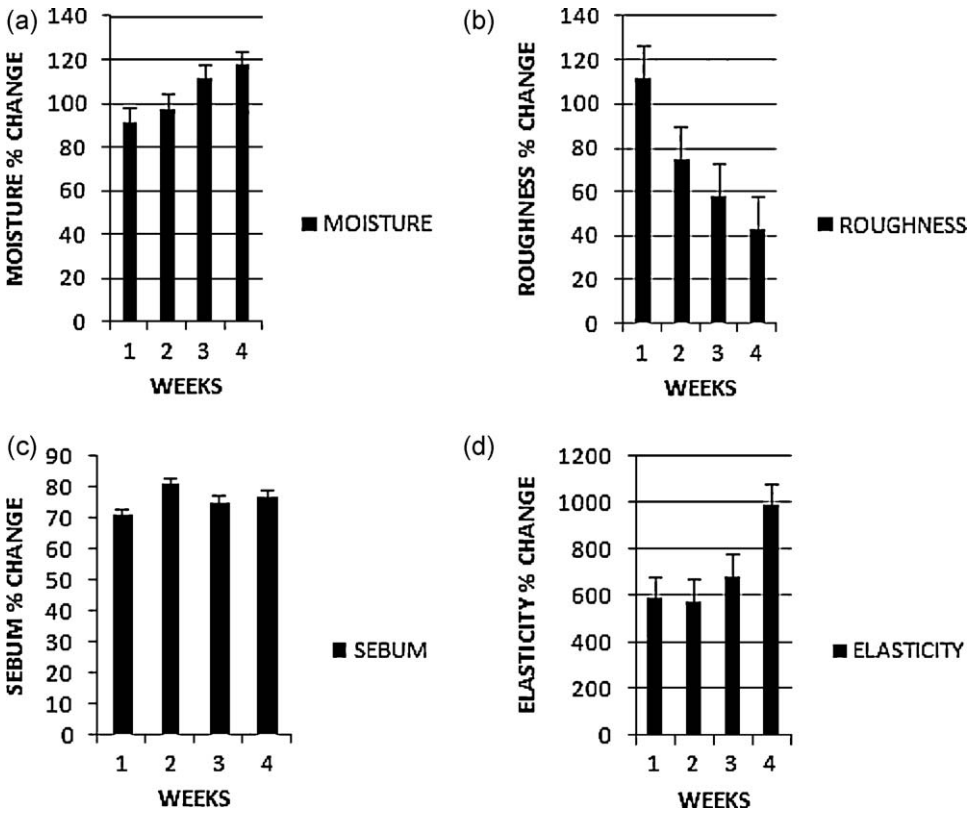

Fig. 7. Changes in skin parameters: (a) skin moisture, (b) skin roughness, (c) skin sebum, (d): skin elasticity.

4. Conclusion

The results justified that VE was incorporated into CS microspheres successfully. Formulation was shown to have an *in vitro* antioxidant effect when evaluated by the DPPH test. According to the *in vitro* release studies, CS microspheres showed burst release at the 5th minute after application, however release lasted for 6 h. *In vivo* studies showed that CS microspheres containing VE is a promising formulation for an antiaging effect due to the decrease in skin roughness and increases in skin moisture and elasticity.

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