Electrospaying of zein for the preparation of micro/nano-particles loaded with sarcopoterium spinosum extract

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ABSTRACT

Natural compounds that are isolated from various plant species have been the focus of folk medicine due to their bioactivities. Pharmaceutical, food and cosmetic industries also utilize these natural compounds with antioxidant and antimicrobial properties. Harsh processing and storage conditions causing loss of their bioactivities limit their applications. Therefore, biopolymERIC carrier systems are required to preserve the natural bioactive compounds stability. Sarcopoterium spinosum is used in folkloric medicine for curing diabetes, gastrointestinal illnesses, cancer and pain. In this study, both solution and process parameters for electrospraying technique were investigated to encapsulate S. spinosum extract including natural bioactive compounds in zein particles. The best results were observed in 5% (w/v) of zein concentration in 70% aqueous ethanol. 0.3 ml/hour flow rate and 14 kV applied voltage were used in order to obtain narrow size distribution and smooth particle structure. The best mixture of S. spinosum extract and zein was achieved at weight ratio of 1:5. The zein microparticles with extract showed remarkable antioxidant activity.

Keywords: zein; electrospray; Sarcopoterium spinosum; encapsulation.

1. INTRODUCTION

Medicinal plants have been used as folkloric medicine for therapeutic usage as regulating blood glucose level, decreasing stress in the molecular level of the cell or to reduce risk of cancer [1-4]. Pharmacological and cosmetic industry also utilizes medicinal plants due to the presence of active substances as they possess antioxidant components known as phytochemicals [5-7]. The role of phytochemicals such as phenolic compounds is to react with inactive free oxygen radical. Antioxidants show function like terminators of free radicals chain. They also act as chelators. They can chelate redox active transition metal ions which can catalyze lipid peroxidation [8, 9]. They inhibit or lag the oxidation of lipids and other molecules. Antioxidants are capable to do that by deliver a hydrogen atom to radicals (R). As a result of this donation they suppress the formation of reactive oxygen species (ROS) [10].

The use of natural phenolic compounds is problematic because they are susceptible to environmental conditions. Processing conditions also cause a bioactivity loss. Permeability, low solubility, loss of stability or storing conditions can be reason in processing. In biological systems before reaching the circulation system, degradation of phenolics in gastrointestinal tract also causes the functionality loss. Those problems limit the area of disposal of these compounds. Plant bioactive chemicals activity depends on preserving their stability [11-14]. Therefore, usage of polyphenols requires a protection layer around it. So that layer can maintain structural unity of the phenolic compounds. Encapsulation has gained great interest as a delivery or carrier system to preserve active agents and enhance their efficacy during application. Encapsulation is a process to enclose active compounds within a protective layer for a period of time [15,16].

Main reasons for encapsulation of plant extracts are to protect them from environmental effects like UV, moisture and oxygen. By encapsulation one can prevent reactions such as dehydration and dehydration oxidation in plant extracts. As a result the shelf life of compounds increase and processing steps improve. Zein is the prolamin fraction of corn protein. It has been investigated as a possible material for its coating capability of bioactive compounds. As a result of its film forming ability, zein has been used in pharmaceutical and food industries. Moreover to its film forming ability, it has also improved stability, potential biodegradability and biocompatibility properties [17, 18]. Examples are exist as zein coated microspheres used for insulin delivery and ciprofloxacin-loaded zein microsphere films, [19, 20] or encapsulated α-tocopherol in zein-chitosan complex [21, 22]. Thus, zein can overcome the disadvantage of the hydrophilic polymeric system to maintain sustained drug release [12]. It can extend the shelf life of phenolic products by avoiding contact between the phenolics and prooxidant factors. Those factors can be listed as oxygen barrier feature, its low water uptake values, high resistance to UV light or temperature [6,23, 24]. Zein particulate structures were identified as safe (GRAS) biopolymers for incorporation into food matrices[25, 26]. Zein particles also commercialized in pharmaceutical industry after approval of FDA[17].

The Sarcopoterium spinosum species is a folkloric medicinal plant in the Mediterranean. According to the ethnobotanical survey, S. spinosum is used in folkloric medicine for the treatment of diabetes, pain, digestive problems and cancer [27,28,29]. Its bioactivity was investigated in our previous study [30] after optimization of extraction process and determination of effects of
2. EXPERIMENTAL SECTION

Acetonitrile (HPLC grade) was obtained from Sigma Aldrich (Steinheim, Germany). Ethanol, acetic acid, phosphoric acid, formic acid, ethylacetate, methanol, tetrahydrofuran were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was obtained from Riedel (Seelze, Germany). ABTS (2,2’-azinobis(3-ethylbenzothiazoline-6-sulphonic acid), zein and standards were purchased from Sigma Aldrich (Steinheim, Germany). Gallic acid was purchased from Merck (Darmstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich (Steinheim, Germany). Folin-ciocalteu reagent was obtained from Merck (Darmstadt, Germany), sodium carbonate anhydrous (99.5%) was purchased from Riedel (Seelze, Germany), Fetal Bovine Serum (FBS), Dulbecco’s Modified Eagle Medium (DMEM), Penicillin–Streptomycin, Phosphate-Buffered Saline (PBS) were obtained from Gibco (New York, USA ).

Preparation of Sarcopoterium spinosum extracts.

Sarcopoterium spinosum, collected from the region in Gülbağçe, İzmir, Turkey before the flowering period. Plants were dried at room temperature and stored at clean dark place. Then dried S. spinosum leaves were grounded. Extraction was carried out in 70% ethanol with solid-liquid ratio of 1: 20, at 180 rpm for 24°C for two hours. Centrifugation of plant extract solution was performed at 5000 rpm for ten minutes in order to remove solid particles. Then followed by vacuum-filtration to remove plant debris. After that step evaporation was done to vaporize ethanol from the solvent and lastly, lyophilization was performed to obtain powder form. Mass yields of the percent extraction (w/w) of plant materials were calculated.

Preparation of Zein Particles via Electrospray.

Zein was dissolved in aqueous ethanol at 60, 70 and 80% (v/v) and stirred at room temperature ranging from 1 to 20% (w/v), until completely dissolved. Prepared zein solutions were fed into a 5ml plastic syringe with a needle having a tip diameter of 0.6 mm. The space between the tip of needleand the target was 10 cm. The particles were deposited on a constant target of aluminum foil under 10, 12, 14 and 18 kV, with a flow rate ranging from 0.15 to 1.5 ml/h. After determination of the effect of the main electrospray factors on the form and size of the zein particles, zein solutions with S. Spinosum extracts were prepared in %70 (v/v) aqueous ethanol. 2:1 , 1:1 , 1:5 , 1:10 , 1:20, 1:50 and 1:100 as extract:zein ratio (w/w).

Scanning Electron Microscopy.

The morphology of zein particles with and without extract was analyzed using scanning electron microscopy (SEM) (Philips XL30 SFEG and Quanta FL ESEM) after gold-palladium sputtering. Images were taken by applying an electron voltage of 5-7 kV. Diameter of particles was measured by processing the images with Image J software. Size distribution of particles was analyzed by minitab 15 software.

Determination of Encapsulation Efficiency.

Obtained S. spinosum extract filled zein microparticles were dissolved in 70% (v/v) aqueous ethanol and measured by spectrophotometer at 350 nm which exhibited no absorption for zein. Loading efficiency was measured by the aid of antioxidant activity of milled microsphere with bead beaker. S. spinosum extract encapsulation efficiency was analyzed by the following equation;

Encapsulation efficiency (%) = (Absorbance of the extract in microsphere x100)/Absorbance of the extract initially added

Determination of Total Antioxidant Capacity.

Encapsulated particles were disintegrated mechanically by bead beater in deionized water and the extract was regained. Aqueous samples were subjected to antioxidant activity assay. Trolox, a standard antioxidant, was used for the standard calibration curve to compare the capability of the extract to scavenge ABTS radical cation [31].

Cell Culture and Determination of Cytotoxic Activity.

NIH3T3 mouse fibroblast cell line was maintained in DMEM supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 100 µg ml-1 streptomycin, 100 U ml-1 penicillin under 5% carbondioxide (CO2) and 95% humidity at 37 °C. Since cytotoxicity of extract loaded zein particles was determined by indirect method, samples were extracted by incubation with culture medium for 24 h. Pre-seeded cells were treated with obtained extract for 24, 48 and 72 h. Cytotoxic activity was determined by MTT assay and absorbances were measured at 545 nm. Cell viability was calculated using the following formula:

Cell viability (%) = (Absorbance value sample / Absorbance value control) x100

3. RESULTS SECTION

Electrospray method was used to obtain S. spinosum extract inserted zein microparticles, the best encapsulation condition for zein was determined in electrospray before the loaded sample was produced. The main factors that affect the structure of the microparticles and the concentration of zein polymer in the solution include viscosity, surface tension, the solvent conductivity and the processing conditions. Those processing conditions can be flow rate of solvent, applied voltage to the needle tip and distance between the needle tip and the type of the collector plate. As a result of that variability, the effect of zein concentration change, solvent concentration and different applied voltage were searched on the dimension and structure change of
the zein microparticles. The investigated zein polymer concentrations were selected as 1, 2.5, 5, 10 and 20 percent in order to optimize the method parameters. The most important factor in electrospray method is polymer concentration. The viscosity and surface tension linked to the concentration of polymer.

Figure 1 shows a change in the morphology of the zein structures which were obtained with increasing zein concentrations. In addition, increasing surface tension with increasing zein concentration causes more instability. This resulted in unbalanced dispersion of particle diameters. Finally, it can be seen in figure 1 that fiber like structure was formed rather than sphere particles with the highest concentration of polymer. As a result of low intermolecular entanglements between polypeptide chains, zein polymer was not able to form a spherical structure after solvent evaporation. According to the SEM image, particles with smooth surface and narrow size distribution were obtained with 5% zein concentration.

Figure 1. SEM images showing the size and shape of microstructures at different zein concentration. Needle distance to collector (10 cm), flow rate (0.3 ml/h) and voltage (14 kV). Concentrations of zein (w/v) in figure A: 1%; figure B: 2.5%; figure C: 5%; figure D: 10%; and figure E: 20%.

In Figure 2 the particle size distribution is given. According to the distribution graph with 5% zein concentration at a voltage of 14 kV and a flow rate of 0.3 ml/h, the electrospraying resulted in an average particle diameter of 200 nm ± 56 nm.

Figure 2. Particle size distribution graph of zein microstructures. Zein concentration (5%), voltage (14 kV), distance between needle and collector (10 cm), flow rate (0.3 ml/h).

The solvent ethanol concentrations were studied by using 60, 70 and 80% aqueous ethanol solutions. The effect of solvent concentration on the particle morphology was shown in Figure 3.

Figure 3. SEM images showing the size and shape of microstructures at different solvent concentration. Flow rate (0.3 ml/h), zein concentration (5%, w/v), voltage (14 kV), distance between needle and collector (10 cm). Solvent concentration (ethanol in dH₂O; v/v) in figures were A: 60%; B: 70%; C: 80%.

In Figure 4 it was observed that if ethanol concentration changed between 60 to 80%, the particle sizes decreased, and perfect spherical shapes were deformed. Rise in ethanol concentration had a small negative effect on the shape of spherical shaped particles. According to the SEM results 70% ethanol solution was defined as optimal solvent system. According to SEM images in Figure 4, there was no effect of low voltage on round shape and narrow size distribution. On the other hand, as the voltage increased the particles tended to form aggregates.

Figure 4. SEM images showing the size and shape of microstructures at different voltages. Zein concentration (5% (w/v)), flow rate (0.3 ml/h) and needle to collector distance (10 cm). Voltages in the figures were; at A: 10kV; at B: 12kV; at C:14 kV; at D:18 kV.

The flow rate is the main factor that affects electrohydrodynamic atomization process. In the electrospray methodology the particle
diameter increases with the flow rate. In Figure 5, the effect of the flow rate on the microparticles can be seen. In order to detect the effects of the flow rates, voltage was fixed at 14kV, zein concentration was fixed to 5% in 70% aqueous ethanol solvent. The flow rates were ranged between 0.15 and 1.5ml/h. As seen in Figure 5, at flow rate of 0.3ml/h the lowest particle diameter was obtained.

Figure 5. SEM images showing the shape and size of microstructures at different flow rates. Zein concentration (5 % (w/v)), voltage (14 kV), distance between needle and collector (10 cm). Flow rates were; A: 0.15 ml/h; B: 0.3 ml/h; C: 0.6 ml/h; D: 1 ml/h; E: 1.5 ml/h.

Zein microparticle coated *S. spinosum* extracts were prepared after the best working conditions were decided. The decision was made with respect to the structure and narrow size distribution of the prepared zein particles. The effects of the plant extract concentration on particle size and structure were investigated. Then, the bioactivities of *S. spinosum* extract loaded zein microstructures were analyzed.

SEM images provide information on the particle morphology and size. The *S. spinosum* extract loaded zein microstructures were formed with 5% (w/v) zein, 70% (v/v) ethanol at a flow rate of 0.3 ml/h. The applied voltage was 14 kV and the needle was put 10 cm away from collector. For the production of *S. spinosum* extract loaded zein microstructures, *S. spinosum* extract was dissolved in 70% aqueous ethanol solution with a zein concentration of 5% (w/v) at several extract to zein weight ratio of 2:1, 1:1, 1:5 1:10, 1:20 and 1:50. The result SEM images for those weight ratios are given in Figure 6. As seen in Figure 6, the morphology of the prepared microstructures was partially spherical at (the extract powder: zein) weight ratio of 2:1 and 1:1, but the image of the microparticles was rough. The reason for this rough structure could be inefficient encapsulation capacity of zein. Because a high amount of *S. spinosum* extract causes inefficient surface tension. The desired morphology with narrow size distribution at high extract loading amount was achieved at a weight ratio of 1:5, as seen in Figure 6-C.

In Figure 7, particle size distribution obtained at a (the extract powder: zein) weight ratio of 1:5 was drawn. The average diameter of *S. spinosum* loaded zein particles was found as 193±57 nm. In addition to particle size, the extract loading efficiency microparticles prepared at the extract to zein ratio of 1:5 was calculated. Encapsulation efficiency was measured as 96 % for the microparticles prepared at 1:5 (extract: zein) weight ratio. Therefore, 1:5 (extract: zein) weight ratio was determined as optimum weight ratio to prepare *S. spinosum* loaded zein microstructures for the rest of this study.

Figure 6. SEM images showing the size and shape of microstructures at different zein concentrations. Flow rate (0.3 ml/h), voltage (14 kV), needle to collector distance (10 cm), zein concentration (5%,w/v). *S. spinosum* extract: zein (w/w), A: 2:1; B: 1:1; C: 1:5; D: 1:10; E: 1:20; F: 1:50.

Particle Size Distribution of *S. spinosum* Extract Loaded Zein Microstructure

As seen in Figure 6, the morphology of the prepared microstructures was partially spherical at (the extract powder: zein) weight ratio of 2:1 and 1:1, but the image of the microparticles was rough. The reason for this rough structure could be inefficient encapsulation capacity of zein. Because a high amount of *S. spinosum* extract causes inefficient surface tension. The desired morphology with narrow size distribution at high extract loading amount was achieved at a weight ratio of 1:5, as seen in Figure 6-C.

In Figure 7, particle size distribution obtained at a (the extract powder: zein) weight ratio of 1:5 was drawn. The average diameter of *S. spinosum* loaded zein particles was found as 193±57 nm. In addition to particle size, the extract loading efficiency microparticles prepared at the extract to zein ratio of 1:5 was calculated. Encapsulation efficiency was measured as 96 % for the microparticles prepared at 1:5 (extract: zein) weight ratio. Therefore, 1:5 (extract: zein) weight ratio was determined as optimum weight ratio to prepare *S. spinosum* loaded zein microstructures for the rest of this study.

Figure 7. Particle size distribution of *S. spinosum* extract loaded zein particles formed at 1:5 extract zein weight ratio.

The *S. spinosum* extract loaded in zein particles that were formed at weight ratio of 1:5 (extract: zein) was investigated for their total antioxidant activity and cytotoxicity. The antioxidant activity of
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4. CONCLUSIONS

In this study encapsulation of S. spinosum extract with zein, the prolamin fraction of corn protein was achieved by using electrospraying method to prepare functional microparticles with antioxidant properties. Electrospraying parameters to encapsulate S. spinosum extract were investigated. By changing method parameters, zein particles were successfully prepared to encapsulate S. spinosum extract with high encapsulation efficiency. Characterization of the prepared microparticles in terms of morphology, antioxidant activities and cytotoxic properties was carried out. The extract loaded zein particles had antioxidant capacity. The study demonstrated that electrospraying process could be a potential new approach for enhancing the extract stability by encapsulation.

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