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Evaluation of Nano and Microcapsules of Silymarin in Simulated Gastrointestinal Conditions for Animal Target Delivery

Research Article

S. Yousefdoost[1], F. Samadi[1*], S.M. Jafari[2], S.S. Ramezanpour[3], F. Ganji[4] and S. Hassani[1]

[1] Department of Animal and Poultry Physiology, Faculty of Animal Science, Gorgan University of Agricultural Science and Natural Resources, Gorgan, Iran
[2] Department of Food Materials and Process Design Engineering, Faculty of Food Science and Technology, Gorgan University of Agricultural Science and Natural Resources, Gorgan, Iran
[3] Department of Plant Breeding and Biotechnology, Faculty of Plant Production, Gorgan University of Agricultural Science and Natural Resources, Gorgan, Iran
[4] Department of Biology, Faculty of Science, Golestan University, Gorgan, Iran

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*Correspondence E-mail: f.samadi@gau.ac.ir

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ABSTRACT

The main goal of this research was to compare the *in vitro* release rates of nano- and microcapsules of *Silybum marianum* extract (SME) in animal simulated gastric and intestinal medium conditions. The extract was encapsulated within sodium alginate carriers using emulsification/internal gelation method. Particle size, zeta potential, polydispersity index (PDI) and morphology of nanocapsules were analysed via dynamic light scattering (DLS) and transmission electron microscopy (TEM). In addition, the effect of ultrasonication on nanocapsule properties and the release profiles of SME-loaded nano/microcapsules were evaluated. Results showed that ultrasonication reduced the size of capsules from 657.5 nm to 169.1 nm which resulted in uniform particles with a low PDI. Encapsulation efficiency for nanocapsules was 61%. Alginate nano/microcapsules protected polyphenols in simulated gastric medium as observed by 10% and 12% release, respectively. Nanocapsules released their contents higher and faster than microcapsules in simulated intestinal fluid ($P<0.05$). In conclusion, alginate nanocapsules containing SME were made successfully with a release rate of over 90% of extract within simulated intestinal medium which can be used for animal target delivery purposes.

KEY WORDS nano/microcapsule, *Silybum marianum*, simulated gastrointestinal conditions, ultrasonication.

INTRODUCTION

A number of bioactive compounds may be degraded during gastrointestinal digestion and therefore need to be protected (McClements, 2015). In addition, the optimal size of nutrients can improve the efficient absorption from the mucosa of gastrointestinal tract. Undesired taste and odor of some foods is another problem which decrease their palatability. These features are important when the goal is consuming unpleasant meals such as fish oil or medical plants extract.

Nanoparticles are matrix systems of a dense polymeric network in which a bioactive compound may be dispersed throughout the matrix. Since nanoparticles are sub-cellular in size, they have versatile advantages for targeted, site-specific delivery purposes as they can penetrate circulating systems and target sites.

Encapsulation involves the coating of a material into another material. Thus, the purpose of encapsulation is to protect its content from the external and internal environmental conditions which could be destructive. Additionally, encap-

sulation improves dissolution rate, cell membrane permeability and bioavailability of low-soluble nutraceuticals (Fang and Bhandari, 2010).

Nanocapsulation, a combination of nanotechnology and capsulation, is defined as the entrapping of active ingredients in nanometer-sized capsules (5 to 1000 nm, generally between 100-500 nm (Mokhtari *et al.* 2017; Mora-Huertas *et al.* 2010; Natrajan *et al.* 2015). Various biocompatible and biodegradable biopolymers including natural polymers like dextran, alginate, chitosan, gelatin, pullulan and hyaluronan or synthetic polymers like poly-dl-lactic-co-glycolic acid (PLGA), polylactide (PLA) and polymethyl methacrylate (PMMA) have been used in the formation of nanoparticles to maximize delivery efficiency and increase the desirable benefits (Mokhtari *et al.* 2017).

Alginate, a natural, non-toxic, biodegradable, low in cost and non-immunogenic substance, is used to encapsulate materials (Pawar and Edgar, 2012). Alginate is linear anionic polysaccharide derived from brown seaweeds and consists of residues of (1, 4)-linked β-D-mannuronic (M) and α-L-guluronic (G) acid in blocks (Figure 1a) [30]. Ionic cross-linking with multivalent cations (e.g. Ca^{2+}) is the most common method for the formation of alginate gels, forming egg-box structure in which Na^{+} from the G blocks exchange with Ca^{2+} (Figure 1b) (Pawar and Edgar, 2012; Poli *et al.* 2008). Because of the mentioned characteristics, alginate has been widely used for particle formation in nano and micro range (Lertsutthiwong *et al.* 2008; Machado *et al.* 2012; Sosnik, 2014; Zhang *et al.* 2014).

Silybum marianum is an annual herb and its extract is believed to be benefit for liver diseases. Commonly, *Silybum marianum* extract is made from the seeds, which contains approximately 4-6% silymarin (Greenlee *et al.* 2007). Silymarin has antioxidant and hepatoprotective properties (Ball and Kowdley, 2005; Köksal *et al.* 2009; Wu *et al.* 2015).

However, this flavanolignan has low aqueous solubility, low bioavailability which results in its low gastrointestinal absorption. To overcome this undesired features, different methods have been applied to improve its bioavailability and solubility in oral applications which can be referred to the silymarin-loaded solid nanoparticles (Cengiz *et al.* 2015; Yang *et al.* 2013), silymarin beta-cyclodextrin complexes (Ghosh *et al.* 2011), microspheres of silymarin (Garg and Gupta, 2010), slymarin liposomes (El-Samaligy *et al.* 2006), nanoemulsion (Parveen *et al.* 2011) and silymarin-loaded nanocapsules with Eudragit RS100 (Das *et al.* 2011). For instance, in a study carried out on silymarin nanoemulsion, faster release, improved bioavailability and better absorption of drug was reported (Parveen *et al.* 2011). Due to few works done with alginate and *Silybum marianum* extract, we encapsulated this extract with alginate biopolymer. In this study, alginate nanoparticles containing *S. marianum* extract were prepared, which are typically required for efficient uptake by cells. Also, owing to different release profile of nano- and micro-capsules, which affect their pharmacokinetics (Kothamasu *et al.* 2012; Zhang *et al.* 2014; Zhang *et al.* 2016), the release rate was investigated. Finally, due to importance of ultrasonication in creating small particles, the impact of ultrasonication on capsules during several times was evaluated.

MATERIALS AND METHODS

Preparation of *Silybum marianum* extract

The seeds of *Silybum marianum* were defatted and extracted with 300 mL petroleum ether using Soxhlet extractor (Behr, Germany) for 16 h. After drying, defatted seeds were placed into Soxhlet extractor, then 300 mL ethanol added and extracted for 8 h. Ethanol of the resulted liquid was evaporated using vacuum drying oven (Binder, Germany).

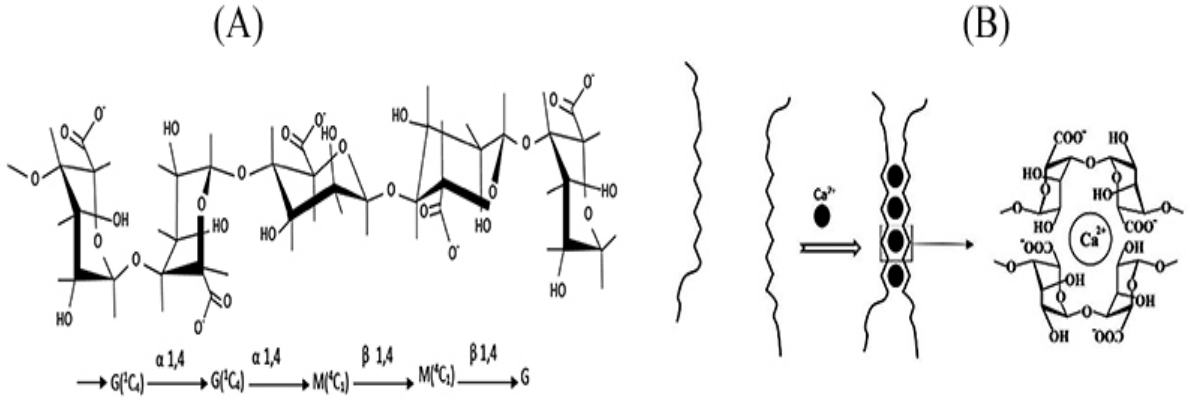

Figure 1 (A) Alginate molecular structure
(B) Formation of an alginate gel by calcium cations, resulting in "egg box" calcium linked junctions

Nanoencapsulation preparation

Alginate nanocapsules were prepared using a modified emulsification/internal gelation method as described previously (Mokhtari *et al*. 2017; Reis *et al.* 2006). Briefly, sodium alginate (Samchun, South Korea) solution (1%, w/v) was prepared by dissolving alginic acid in distilled water for 30 min. Sodium alginate W/O emulsion was made by adding of some alginate solution into a liquid containing Tween 80 (Sigma-Aldrich, USA), diluted extract and canola oil under continuous mechanical stirring at room temperature. After ultrasonication (250 W, 20 kHz, 25 ˚C; UP400E, topsonic, Iran), an appropriate volume of $CaCl_2$ (0.1 M) (Merk, Germany) as a crosslinker was then added into the resulting emulsion and stirred for an additional 10 min. Centrifugation (26064 × *g*, 10 min, 4 ˚C; SIGMA 3K30, Germany) was done and nanocapsule sediments were obtained. Microcapsules were also synthesized using the same protocol with different values (Figure 2).

***In vitro* characterization of encapsulation**

Total phenolic contents

Total polyphenols were determined by Folin-Ciocalteu method (Singleton *et al.* 1999). Aliquots (20 µL) of extracts were transferred into the test tube and its volume increased by adding 1.16 mL distilled water, 100 µL Folin-Ciocalteu reagent and 300 µL sodium carbonate solution 20%. Tube was vortexed, kept in water bath in 40 ˚C for 30 min and absorbance of blue colored mixture recorded at 760 nm. Polyphenols was calculated as a Gallic acid equivalent via the linear equation (1) based on the calibration curve and expressed as mg Gallic acid per mg dry material.

$$Y = 0.0053\ X - 0.0018$$
$$R^2 = 0.99 \qquad (1)$$

Where:

Y: absorbance at 760 nm and X is the concentration of total phenol.

Encapsulation efficiency

To determine encapsulation efficiency (EE), total phenolic contents were used as described by (González-Paredes *et al.* 2011; Harris *et al.* 2011). The amount of bioactive compound entrapped in nanocapsules was calculated using equation (2):

Encapsulation efficiency (EE%)= (total phenolic compounds in supernatant/total phenolic compounds) × 100

The amount of free phenolic compound was obtained from supernatant after centrifugation (26000×g, 10 min, 4 ˚C).

Particle size and zeta-potential

The average size and zeta potential of alginate capsules were measured on fresh samples by a commercial instrument capable of electrophoresis and dynamic light scattering (DLS) measurements (zetasizer Nano-ZS90, Malvern Instruments, Worcester, UK), using 10-mW Helium-Neon laser of 633 nm. First, samples were diluted 1:400 (using double distilled water) in order to prevent multiple scattering effects in size measurement. The morphologies of capsules were evaluated using transmission electron microscopy (Zeiss, Germany).

***In vitro* gastric and intestinal digestion**

Simulated gastric and intestinal fluids were prepared according to method described by (Guzman-Villanueva *et al.* 2013; Zhang *et al.* 2014). Simulated gastric fluid (SGF) was prepared by dissolving 3.2 g of pepsin (Sigma-Aldrich, USA) in 2 g of NaCl in 1 liter deionized water and then pH was adjusted to 1.5 using 1 M HCl. The components of simulated intestinal fluid (SIF) were 10 g/L of pancreatin (Sigma-Aldrich, USA) and 0.05 mol/L of KH_2PO_4 (Merck, Germany) at pH=7.4. Briefly, 0.1 g of wet capsule was added to 9 mL of SGF and then incubated at 37 ˚C for 1 h with constant shaking at 300 rpm. Thereafter, the supernatant was extracted following centrifugation (2000 × *g* for 30 min) at room temperature. For *in vitro* intestinal digestion, 20 mL of SIF was added to the final product of gastric digestion by adjusting the pH to 6.5 using 1M NaOH. The mixture was incubated at 37 ˚C for 2 h with shaking at 300 rpm, during which 5 test tubes were removed at 30 min intervals and centrifuged (1000×g, 15 min) to obtain supernatants.

Statistical analysis

A repeated measure design was used to analyze the data using proc MIXED of SAS (2002) software. Least square means for treatments (nano- and microcapsule) and sampling times were compared by Tukey-Kramer test at 5 percent probability level.

RESULTS AND DISCUSSION

Distribution and size of nano and microcapsules

The average particle size and size distribution obtained by DLS are shown in Figure 3. DLS results indicated average particle size of 958.9 nm and 657.5 nm for microcapsules and nanocapsules, respectively (Figure 3A, B). Also, DLS result for microcapsules showed that the highest number of particles (88.3%) was in the range of 164.2 to 955.4 nm and the remaining amount was above 1000 nm. In order to investigate the effect of biopolymer concentration, none of these two particles was used by the ultrasonic device.

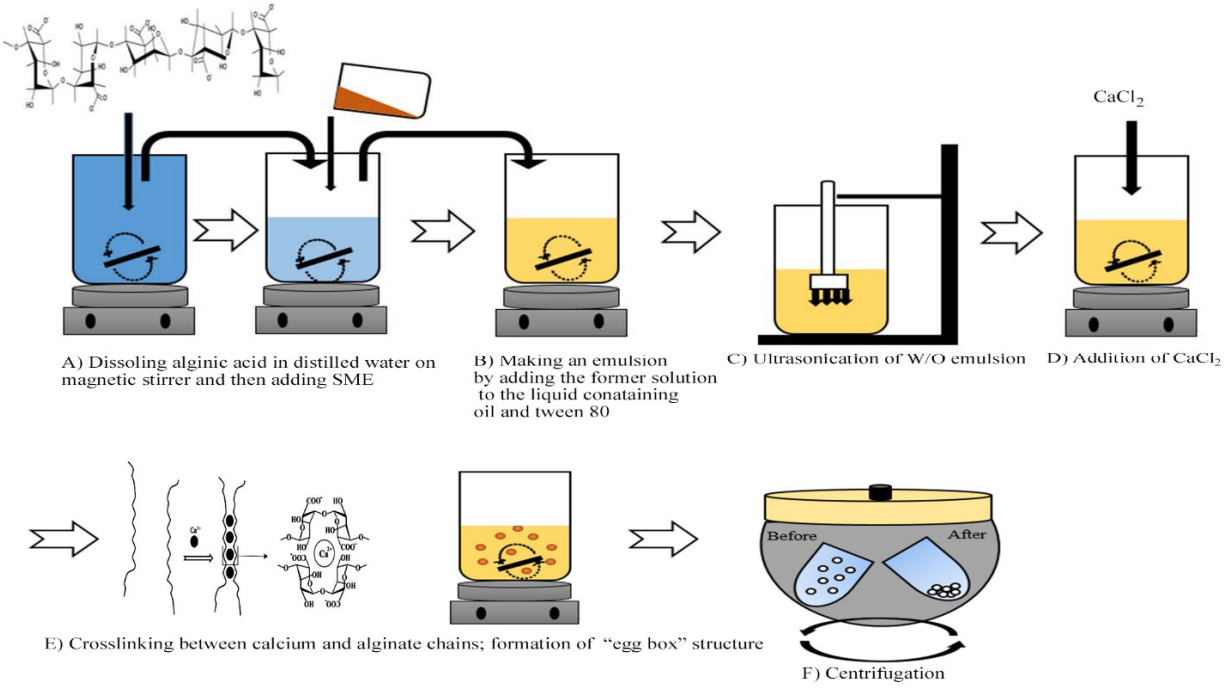

Figure 2 Schematic representation of SME-loaded alginate nanocapsules
SME: *Silybum marianum* extract

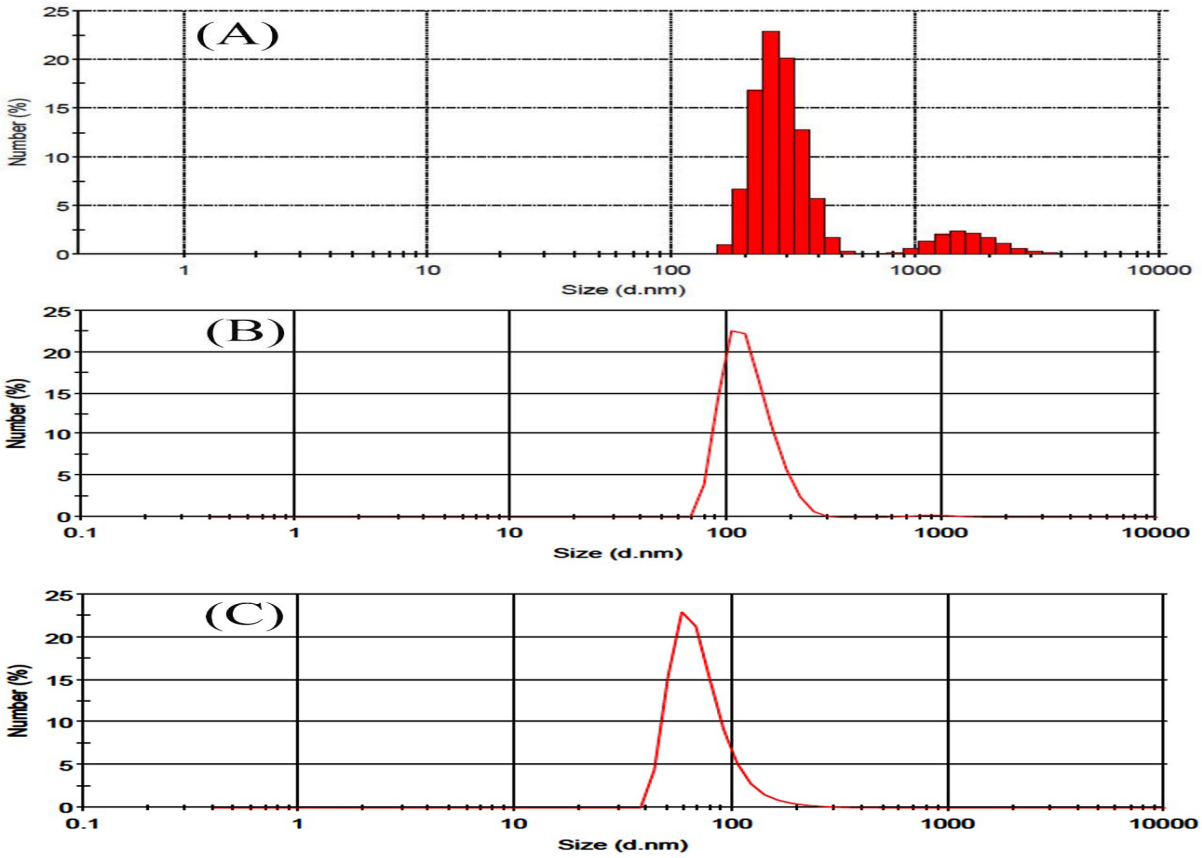

Figure 3 Size distribution of microcapsule (A) and nanocapsules without ultrasonication (B) and with 15 minutes ultrasonication (C) obtained by DLS

The biopolymer concentration has a vital role on the capsule size by affecting viscosity and rigidity of alginate particles which result in the bigger particles. Therefore, the greater particle size which could be associated to the biopolymer concentrations used for microcapsules (1.5% w/v) and nanocapsules (1.0% w/v) is consistent with other reports (Ghayempour and Mortazavi, 2015; Liu *et al.* 2004; Mokhtari *et al*. 2017; Pagar and Vavia, 2013).

The average particle size of nanocapsules without and with sonication were 657.5 and 169.1 nm, respectively (Figure 3B, C). Ultrasonic processors are used as homogenizers to produce small particles in a liquid to improve uniformity and stability. An ultrasonic homogenizer applies a shear stress to break particles into smaller ones in which sonication time plays an important role (Khavari, 2010). It is known that the size of the capsules formed is related to the size of the particles in the first emulsion. Therefore, all the parameters that influence the droplet size in the emulsion affect the particle size in the final emulsion/suspension.

To reduce particle size, homogenization at 8000 rpm (Balcão *et al.* 2013) and magnetic stirring at 1600 rpm for 15 min (Reis *et al.* 2008) were done. Ghayempour and Mortazavi (2015) investigated three types of devices and found that smaller and more stable nanocapsules were obtained using ultrasonic stirrer than those formed by the laboratory reactor and the mechanical stirrer. Lertsutthiwong *et al*. (2008) used alginate to encapsulate turmeric oil and showed that smaller nanocapsules with increasing ultrasonic time were formed.

It was shown that 60 min sonication increased the distribution of smaller particles in compared with 15 min (Poli *et al*. 2008). In this step, an ultrasonic homogenizer with 250 W and 20 KHz (UP400E, topsonic, Iran) was used to produce nano scale particles. As shown in Figure 3 B and C, ultrasonication has changed the particle size distribution. In other words, ultrasonication created many smaller droplets and dispersed them in the fluid. When ultrasonic processors are used as homogenizers, the objective is to break down big particles in a liquid to improve uniformity and stability. A reduction in the mean diameter of the particles increases the number of individual particles. This leads to a reduction of the average particle diameter and increases the particle surface area. On the phenomenon of cavitation, ultrasonic wave propagating in the liquid medium has the ability to generate a bubble or cavity in the liquid medium (Patil and Pandit, 2007). Therefore, ultrasonication is very efficient stage to reduce particles size in dispersions and emulsions. Our results are in agreement with previous works (Ghayempour and Mortazavi, 2015; Lertsutthiwong *et al*. 2008).

Zeta potential of nanocapsules

There are various factors affecting zeta potential including the chemical nature of the polymer, the chemical nature of the stabilizing agent and pH of the medium (Mora-Huertas *et al*. 2010; Surh *et al.* 2006).

The charge of the polysaccharide is in direct relation with zeta-potential of the solution. Some studies showed that polysaccharide concentration significantly affected zeta-potential and anionic structure of sodium alginate resulted in the negative values of zeta potential. In addition, the effect of pH on zeta potential value has been reported in a study on pectin-whey protein nano-complexes (Ghasemi *et al.* 2017).

According to Natrajan *et al*. (2015), zeta potential values less than -30 mV and greater than +30 mV are considered stable. In the current study, we did not observe any aggregation which can be attributed to the high negative zeta potential value. Table 1 shows the zeta potential values of nanocapsules during different sonication times. Our results indicated zeta potential values ranging from -6.09 to -25.0 mV, suggesting the effect of sonication time on the surface properties of the nanocapsules. By ultrasonication, small particles (alginate nanocapsules) with greater surface area expose more anionic groups to the medium which results in high negative zeta potential values. Generally, when all the particles have a large positive or negative zeta potential, they will repel each other followed by a higher dispersion stability.

Polydispersity index is a dimensionless measure of the heterogeneity of particles size in a mixture and is used to show distribution pattern of particles. The values less than 0.25 showing a narrow size distribution with uniform particles, whilst the values greater than 0.5 are considered as a splay distribution (De Sousa Lobato *et al.* 2013). As shown in Table 1, polydispersity index decreases from 0.60 to 0.31 by increasing sonication time, indicating the positive effect of ultrasonic time to form similar particles size. Due to polydispersity index is an indicator of aggregation in the particles, the higher values show a polydisperse system whilst the lower values show monodisperse system. Polydispersed particles have a great tendency to aggregation than monodispersed systems. The present study revealed that increasing sonication time resulted in uniform small particles and lower polydispersity index which prevented particle aggregation, as it was confirmed by TEM image (Figure 4). As the TEM image shows all alginate nanocapules had spherical and non-aggregated structures.

Encapsulation efficiency

Encapsulation efficiency shows the amount of core material encapsulated inside the particles.

Table 1 Effect of ultrasonication time on average size, zeta-potential and polydispersity index of alginate nanocapsules

Sonication time (min)	Average size (nm)	Zeta potential (mV)	Polydispersity index
0	657.5	-6.09	0.60
1	355.2	-20.30	0.46
5	307.9	-22.30	0.35
15	169.1	-25.00	0.31

In the current study, encapsulation efficiency for particles of alginate nanocapsule was 61% which was lower than reported value (71%) by (Reis *et al*. 2006). Efficiency of encapsulation of the bioactive compounds can be affected by the size and/or specific surface areas of nanoparticles, kind and composition of wall material used and methods of encapsulation (Ghorbanzade *et al.* 2017; Klinkesorn *et al.* 2006; Mokhtari *et al*. 2017; Mora-Huertas *et al*. 2010). For example, Mokhtari *et al*. (2017) showed lower encapsulation efficiency, using the internal gelation method. Generally, it has been reported that the reduction of particles size, which represents an increased stability, results in better encapsulation efficiency and therefore a greater preservation of bioactive substances.

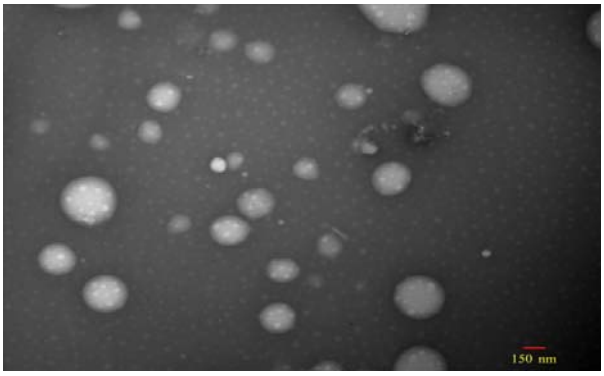

Figure 4 Morphology and size of SME-loaded alginate nanocapsules prepared with 15 min of ultrasonication by TEM

Release profile of nano- and microcapsules

Another goal of this research was to identify if there is a discrepancy between nanocapsule and microcapsule in the core material release. We noticed that the amount of total phenolic compounds releases from nano- and microcapsules was 10% and 12%, respectively, in simulated gastric fluid (SGF) for 1 hour. Zhang *et al*. (2014) reported that release rate of carvacrol in SGF was 4.5% and 1.3% for small and large microcapsules. In another studies, researchers observed carvacrol release in the range of 8 to 11% and 20%, respectively (Wang *et al.* 2009; Zhang *et al*. 2016). The reason for these low release values is because alginate layer shrinks under acidic conditions and the entrapped material cannot easily move out of the capsule (Zhang *et al*. 2014).

At low pH, carboxyl groups of alginate are in the form of –COOH and H-bonding built by –COOH results in heavy interactions between polymer chains. This action reduces the electrostatic repulsion (Deng *et al.* 2010). In both of alginate nano and microcapsules, the release of antioxidant in acidic medium was delayed in accordance with Sosnik (2014) who reported alginate gels are sensitive to neutral and basic pH than acidic conditions. Based on the results mentioned above, the amount of bioactive release in SGF is acceptable.

Leaving these particles in simulated intestinal fluid (SIF) for two hours caused to release of 95% and 81% of total phenolic compounds for nano- and microcapsules, respectively ($P<0.05$, Figure 5), indicating high pH has digestive effect on alginate layer. This pH activates degradation mechanisms of alginate capsules which influence the release of the antioxidant entrapped. In neutral or alkaline media, the carboxyl groups become ionized ($-COO^-$) which, unlike acidic media, electrostatic repulsion changes alginate chain arrangements and swelling of the shell occurs (Deng *et al*. 2010; Sosnik, 2014).

In addition to the capsule type, time was another parameter affected release rate. Table 2 illustrates that in 120 min greatest amounts of phenol released from two capsules ($P<0.05$). This shows a good feature because the aim of encapsulating is to liberate the substantial amounts of beneficial material in suitable time and place. It is well known that efficient absorption occurs in distal parts of gastrointestinal tract. Despite the microcapsules released a high amount of their contents during 2 h, nanocapsules did better ($P<0.05$, Figure 5). There is a condition called "size effect" which says that with decreasing particle size, the solid particles generally tend to show different properties from the bulk material.

Researchers showed that small size microcapsules had higher release rate than large size microcapsules (Zhang *et al*. 2014; Zhang *et al*. 2016). Erdinc *et al*. (2007) indicated that large chitosan-alginate particles had slower release rate (Erdinc *et al.* 2007). Our results are consistent with (Zhang *et al*. 2014; Zhang *et al*. 2016). In line with Zhang *et al*. (2014), releasing of phenolic compounds after 60 min gradually increased and most of them were released at the end of incubation time.

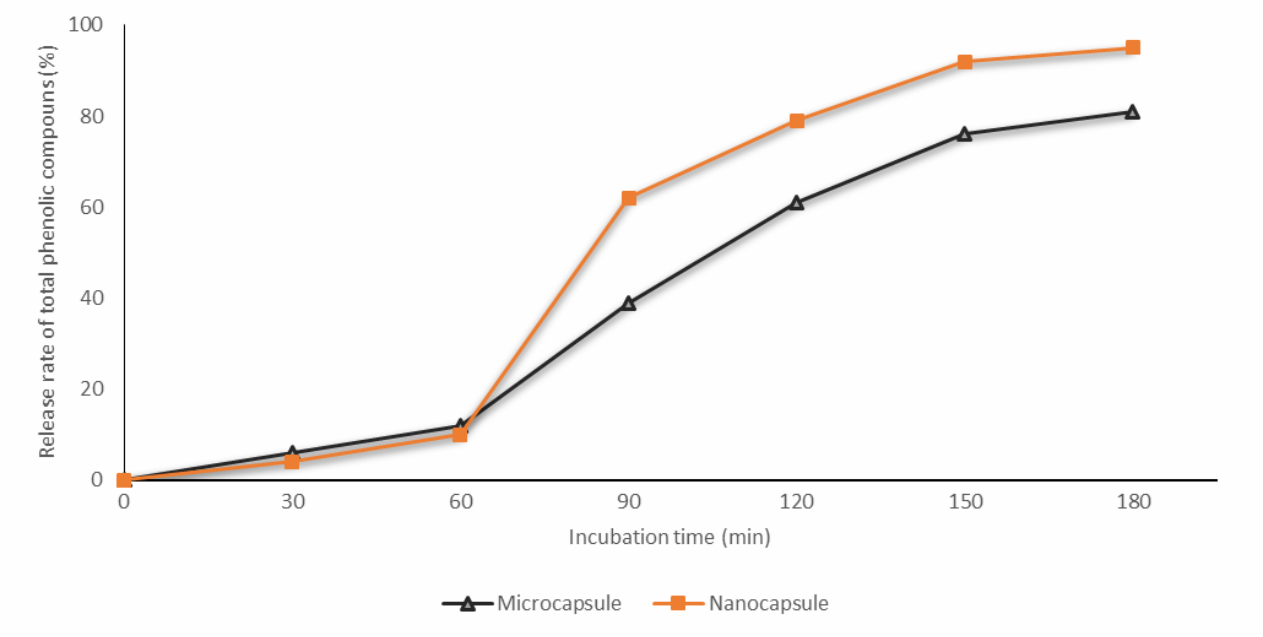

Figure 5 Total phenolic compounds release profiles of nano- and microcapsules in simulated intestinal fluid
SGF: simulated gastric fluid
SIF: simulated intestinal fluid

Table 2 Least square means of release rate of nanocapsules and microcapsules in simulates intestinal fluid

Capsule	Nanocapsule	38.57[a]
	Microcapsule	32.95[b]
	SEM	0.57
	Probability level	0.0022
Time	60 min	9.48[e]
	90 min	15.70[d]
	120 min	24.88[c]
	150 min	59.20[b]
	180 min	69.55[a]
	SEM	1.77
	Probability level	< 0.0001
Capsule × time	Probability level	0.49

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

The release rate of bioactive compounds from microparticles is relatively slower than those of nanoparticles because of the higher surface area and smaller size of nanoparticles (Wang *et al*. 2009; Wu *et al*. 2015). As the micronization of solid particles, the specific surface area and particle size move in reverse directions, i.e. the former decreases and the latter one increases. Due to the presence of therapeutic agents near to the surface area as well as greater surface area of smaller particle, the core material can diffuse instantly than those are in large particles (Kothamasu *et al*. 2012).

CONCLUSION

This study demonstrated that using ultrasound to produce *Silybum marianum* extract-loaded nanocapsules with alginate has potential to protect entrapped material in acidic medium and could be used as delivery carrier of phenolic compounds to the posterior sections of the chicken gastrointestinal tract.

ACKNOWLEDGEMENT

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