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Original Article

IMPROVING THE STABILITY OF BETANIN IN BEETROOT (*BETA VULGARIS,* LINN) EXTRACT USING CHITOSAN MICROPARTICLE

ANITA SUKMAWATI*, CLARA CENDERA MARTHADILLA, ISNA VIRA RISDIYANTI

Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, Sukoharjo, Indonesia
***Corresponding author: Anita Sukmawati; *Email: anita.sukmawati@ums.ac.id**

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ABSTRACT

Objective: The objective of this research is to investigate the ability of chitosan microparticle for maintaining stability of betanin in beetroot (*Beta vulgaris*, Linn) extract. Furthermore, the effect of chitosan concentration during manufacturing of microparticle also explored toward physical stability of active substance in the microparticle.

Methods: Chitosan microparticles were created utilizing the ionic gelation process by employing various concentrations of chitosan (0.5, 1 and 2% *w/v*) and incorporating beetroot extract as the active ingredients. Physical characterization was done including Scanning Electron Microscope (SEM) imaging of microparticles, Drug Loading (DL), and Encapsulation Efficiency (EE). Microparticle stability was evaluated every week on the betanin level and colour (at 40 °C for 28 d).

Results: The outcome shown that the physical properties and EE of beetroot extract in microparticles were significantly impacted by the chitosan concentration during microparticle preparation. The highest EE was found in the microparticle prepared from chitosan 1% (92.1 %). The reducing of betanin level and colour-changed can during storage for 28 d can be diminished by the chitosan microparticles.

Conclusion: The chitosan microparticle has the capacity to prevent betanin from degrading. However, the chitosan concentration during the manufacture of the microparticles had a significant impact on the physical characteristics, loading capacity and ability to inhibit the degradation of the betanin as the active ingredient. The best protection was found in the microparticle prepared from chitosan 1%.

Keywords: *Beta vulgaris*, Linn, Betanin, Chitosan, Microparticle, Stability

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INTRODUCTION

Beetroot (*Beta vulgaris* Linn) is a plant with a high source of antioxidants as it contains the pigment betanin, which is a combination of betacyanin (red-purple) and betaxanthin (yellow). The most largest part of betacyanin is betanin, which is approved by Food and Drug Administration (FDA) for food colorant [1, 2]. This compound is water soluble and is easily degraded due to the influence of pH, temperature, and light [3]. It is an obstacle to maintain the stability of its active substance, which has functions as an antioxidant. Therefore, is necessary to design an active substance delivery system that can effectively protect the stability of betanin for long term-use using microparticles.

Microparticle as a pharmaceutical dosage form, offers an advantage to protect and maintain the active substances from factors that can cause damages, including temperature, humidity, oxygen and microorganism [4]. During microparticle preparation, polymers is required as a matrix for encapsulation. Previous research for maintaining the colour of beetroot extract was carried out using maltodextrin as a matrix for encapsulation. However, the further investigation is needed in order to ensure the encapsulation process of active substances in microparticle as the form of particle is in amorphous flake [5]. The improving of beetroot encapsulation was done by combining maltodextrin and xanthan gum as a matrix and showed good stability at room temperature [6]. The other research indicated that the colour of encapsulated beetroot powder did not showed significant differences after 8 w of storage [7].

In this research, chitosan had been used as a matrix for beetroot juice extract encapsulation. Chitosan has several advantages including hydrophilic polymer, good mucoadhesive properties, low toxicity, biodegradable, good mechanical strength and has ability to improve penetration across mucosal barrier [8–11]. In addition, chitosan has potential for further modification due to presence of amino and hydroxy reactive group and can be applied for controlled delivery of natural compounds as evaluated in previous research by Samprasit [12–14]. The ability of chitosan as an encapsulation matrix for improving stability of active substance was found in research conducted by Rojas (2015) using vitamin C as an active ingredient [15]. The other research showed that the stability of olmesartan medoxomil can be maintained for 2 mo using chitosan as a matrix for encapsulation [16]. In addition, chitosan as a matrix also had ability to inhibit degradation process related to its antibacterial activity [17]. To obtain an expected Encapsulation Efficiency (EE) of active substance, the matrix concentration used during microparticle preparation needs to be explored.

The escalating of concentration of matrix solution during microparticle preparation related to the increasing of EE in microparticle as the high concentration of polymer would induce the microparticle hardening [18]. Therefore, the impact of various chitosan concentrations in solution during microparticle need to be explored related to the physical characteristics and the ability of chitosan microparticle to maintain betanin stability as an active substance in beetroot juice extract.

MATERIALS AND METHODS

Materials

The materials used for microparticle preparation including chitosan (MW 100-200 kDa, from CV Chi Multiguna, Indonesia), sodium tripolyphosphate, acetic acid glacial, tween 80, betanine (Aldrich) and distilled water. All materials were provided by Bratachem, Indonesia unless otherwise stated. The beetroot dried extract was obtained by freeze-drying the beetroot juice in 1% citric acid. The beetroot was identified by determination the plant in Laboratory of Biology, Faculty of Education, Universitas Muhammadiyah Surakarta, Indonesia.

Beetroot dry extract preparation

The beetroot had been cleaned, peeled, and cut into smaller pieces for 200 g was mashed in a blender with 200 ml of 1% citric acid. Afterward, the beetroot juice was separated from the pulp using a clean cotton cloth and dried for four days in a freeze-dried. This

method produced beetroot powder and kept in the refrigerator. The material then used for microparticles preparation.

Formulation of beetroot microparticle using chitosan

Three different chitosan concentrations were used for the preparation of microparticle containing beetroot extract. Chitosan concentrations of 0.5%, 1%, and 2% *w/v* were obtained by dissolving 1.25 g of chitosan in 250 ml, 125 ml, and 62.5 ml of 1% acetic acid, respectively. The mixture was agitated using a magnetic stirrer for two hours at a speed of 700 rpm until solution became clear. For each formula, 0.625 g of beetroot powder that served as the active ingredient was dissolved in 5 ml of distilled water before being added to the chitosan solution and stirred continuously at 350 rpm for 10 min. The mixture was then given Tween 80 at 0.2% *v/v* concentration to stabilize the particles in solution. A 10 ml of 1% *w/v* sodium tripolyphosphate then was added dropwise to each formula to act as crosslinking agent. During 24 h, the mixture was continually agitated at 350 rpm to perfect the crosslinking process. The particle was then precipitated by centrifuging the solution for 15 min at 3000 rpm. The resulting particle was then rinsed three times with 3 ml of distilled water. The wet particle was then dried in a freeze drier for three days before being placed in an aluminium foil-covered container and kept at 4 °C.

Characterisation of beetroot microparticle

Evaluation of the shape of microparticles was carried out using a Scanning Electron Microscope (SEM) while the Drug Loading (DL) and Encapsulation Efficiency (EE) were evaluated by direct method using a spectrophotometer. The pictures of gold-coated microparticles were taken using a scanning electron microscope (JEOL J5M T300) to observe the shape of the particles.

With the intention of determine the entrapment of betanin in chitosan microparticle, a 50 mg of microparticle were dissolved in 1 ml of 1% acetic acid before being combined in a 1:4 ratio with 4 ml of purified water. To precipitate the chitosan debris, the solution was centrifuged at 3000 rpm for 5 min. A UV-Vis spectrophotometer (Genesys 10S) was used to measure the absorbance of clear solution at 532 nm to calculate the amount of betanin using calibration curve Y = 0.0002 x-0,0325. Equations (1) and (2) were used to estimate EE and DL of betanin inside chitosan MP.

$$\text{EE } (\%) = \frac{\text{amount of betanin in microparticles}}{\text{amount of extract used in microparticles preparation}} \text{x } 100\% \text{..... eq. [1]}$$

$$\text{DL } \left(\%\frac{\text{w}}{\text{w}}\right) = \frac{\text{amount of betanin in microparticles}}{\text{amount of sample microparticle}} \text{x } 100\% \text{ ……eq. [2]}$$

Evaluation of betanin stability and colour-changed in microparticles

A 50 mg of microparticle containing beetroot extract made from chitosan concentration of 0.5%, 1% and 2% were weighed and kept in a climatic-chamber for 28 d at 40±2 °C and relative humidity 75±5%. The 50 mg of beetroot dry extract was kept underwent the same condition. Regular sampling every 7 d interval used to monitor the betanin level in the chitosan microparticles and beetroot dry extract from the start of the experiment through day 28th. The level of betanin in the samples was evaluated by dissolved the samples in each timing point using 1 ml acetic acid 1%. The solution then diluted with 4 ml distilled water to dissolve betanin from samples. The dissolution process of chitosan microparticles were conducted inside an ultrasonicator bath (Branson 1510) for 15 min. The chitosan debris from microparticles were separated from betanin solution by centrifugation for 5 min. The betanin absorbance in supernatant then measured using UV-Vis Spectrophotometer (Genesys TM 10S) at λ 532 nm. The betanin level in microparticles and beetroot dry extract then calculated using standard betanin calibration curve.

The color-changed in microparticle was evaluated by observing changes in the colour of samples of chitosan microparticles containing beetroot at the similar time points as betanin level evaluation. Observations were made by taking pictures of the samples using camera (Vivo V5 lite mobile phone) without magnification.

RESULTS AND DISCUSSION

Beetroot microparticle preparation and characterization

The betanin components in beetroot are sensitive to changes in temperature, pH and light; therefore, the citric acid solution was utilized to keep the active substances stable. Citric acid solution was used to maintain pH stability and colour of beetroot product due to its lower pH level would reduce the likelihood of enzymatic browning. As consequences, it would preserve colour and stability of beetroot in dry extract [19].

Ionic gelation method was used for microparticle preparation. In this approach, the polyelectrolytes undergo a cross-linking process while their multivalent ion pairs are present. Chitosan was dissolved in 1% acetic acid using a protonation reaction mechanism. The amine group absorbed the H^+ that produced by acetic acid and making it became positively charged (NH_3^+). The dissolution of the chitosan in acetic acid solution is brought on by the creation of these ions. The crosslink process take place when the negatively charged of tripolyphosphate polyanion's group forms a complicated interaction with the positively charged amine group [20].

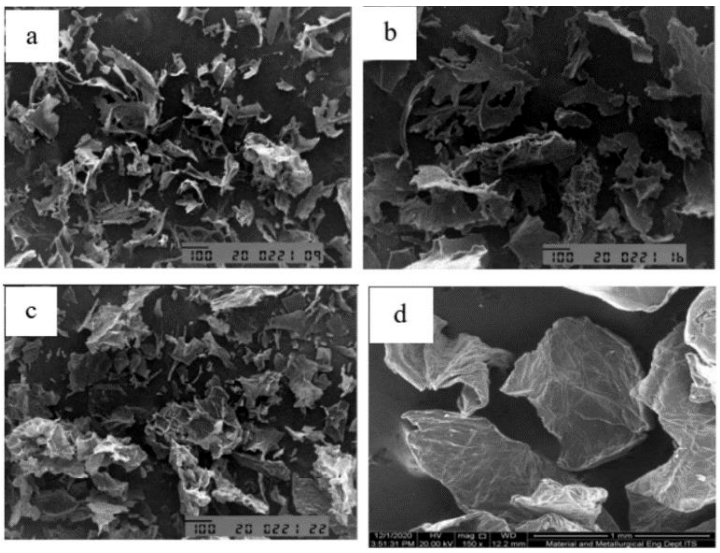

Fig. 1: Chitosan microparticle containing beetroot extract using chitosan concentration of 0.5% (a), 1% (b) and 2% (c) during microparticle preparation and the original chitosan (d). The SEM images was taken with 100 times magnification except for original chitosan (150 times). The scale is in µm

The morphology of beetroot microparticles were carried out by observing using SEM. The result showed that the shape of chitosan microparticles containing beetroot extract were not in spherical but shrunken-flake form with wavy surface (fig. 1). The flakes was formed might related to the shrinkage of particle due to rapid solidification of particles [21]. A quick solidification particle wa occurred as the high sodium tripolyphosphate concentration (1%) was used for MP preparation. However, the morphology of chitosan microparticles containing beetroot showed the significant difference from original chitosan. It is indicated that the ionic gelation method changed the structure of original chitosan. It can be seen from fig. 1c that the chitosan-2% microparticle produce larger size of particle and more solid particle compared to chitosan-1% and chitosan-0.5% microparticle. When the high concentration of chitosan was used, the cross-linking between chitosan and tripolyphosphate occurred more rapidly. As a result, the particle tend to aggregate to form larger particle size [22]. This phenomenon also reported by Sreekumar *et al.* [23].

The determination of DL and encapsulation efficiency EE was carried out as a baseline for calculated the levels of active substances in the microparticles during the stability test. The amount of betanin inside the chitosan microparticle was demonstrated by DL, while the effectiveness of the entrapment of betanin inside chitosan microparticle was described by EE.

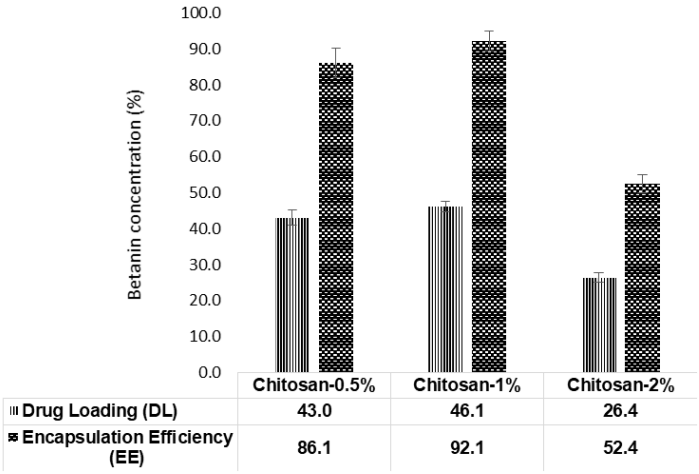

Fig. 2: The drug loading (DL, %*w/w*) and encapsulation efficiency (EE, %) of betanin inside the chitosan microparticle using 0.5, 1 and 2 % *w/w* chitosan solution during manufacturing (n=3), bars represent SD

Fig. 2 indicated that the chitosan-1% microparticle and chitosan-2% microparticle had the highest and the lowest DL, respectively. This result is contradictory with Joshi *et al.* claim [24] where increasing polymer concentration will improve polymer's capacity to cross link and bind the active ingredient, leading to increased drug loading and encapsulation efficiency. However, Ko *et al.* [25] stated that the high viscosity of chitosan solution caused by high concentration of chitosan in the formulation would generated sturdy and thick microparticle walls, therefore reduce capacity of chitosan to swell and adsorb the active component. As a result, the beetroot extracts only absorbed partially inside the microparticle.

Betanin stability and color-changed in microparticle

Betanin's stability in chitosan microparticle and dried beetroot extract was assessed for 28 d at a temperature of 40±2 °C and Relative Humidity (RH) of 75%±5%. This condition was applied in accordance with the World Health Organization (WHO) recommendations for accelerated stability testing of finished pharmaceutical product [26]. The amount of betanin in microparticles and dry extract was assessed at the start of trial (day 0) and until day 28 of the experiment. From day 7 to day 28, the amount of betanin gradually decreased in all microparticle and dry beetroot extract formulations. Based on the amount of betanin inside microparticle or in the beetroot dry extract at day 0, the percentage of reduction in the betanin level was calculated. The dried beetroot extract demonstrated the largest reduction in amount of betanin (34.4%) after 28 d of storage as shown in fig. 3 while the percentage reduction of betanin level in chitosan microparticles varied from 5.6%-27.4% depend on chitosan concentration during the microparticle preparation.

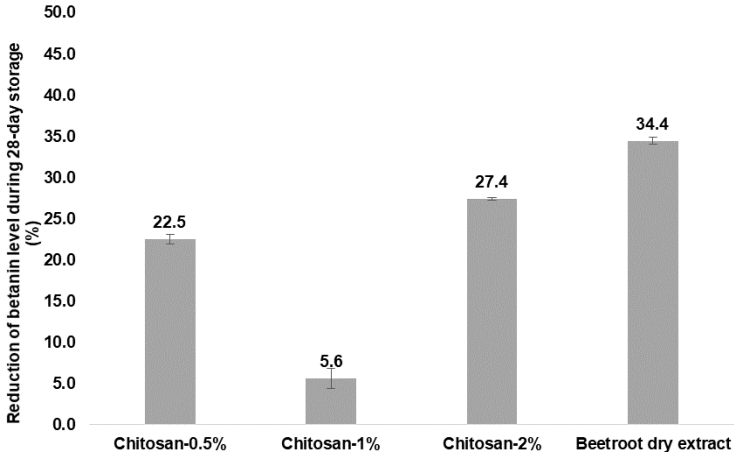

Fig. 3: Betanin level reduction in chitosan microparticles and beetroot dry extract during the storage of 28 d at 40±2 °C, RH 75%±5% (n=3)

The lowest percentage of reduction in betanin level was found in chitosan-1% microparticle (fig. 3); therefore it is clear that chitosan-1% was the most stable microparticle compared to chitosan-0.5% and chitosan-2% in terms of protection of betanin inside the microparticle. However, the chitosan-0.5% microparticle had significant higher protection of betanin level compared to chitosan-2% ($p<0.05$). The capacity of betanin to be trapped on chitosan microparticles can be improved by increasing the concentration of chitosan as a microparticle matrix to 1%. However, boosting the chitosan content to 2% would induce formation of tighter gel pores. Consequently, the permeability of active substance to entrap inside the matrix was getting smaller and it not well-absorbed inside the chitosan matrix. This hypothesis also supported by the lowest DL of chitosan-2% as described in fig. 2. The same behaviour was observed in Li's research [27], which demonstrated a decrease in chitosan's capacity to scavenge free radicals with an increase in chitosan concentration at a certain concentration.

The colour change stability test on the chitosan microparticle was observed at the similar time point with the stability test fig. 4 showed some photos related to colour change in the samples at day 0, 14 and 28. On day 14, chitosan-0.5% microparticle could maintain its colour. However, on day-28, the colour of MP started to shift from red to a faintly yellowish. The colour of chitosan-1% MP did not significantly change or tended to remain consistent from day 0 to day 28. On contrary, the chitosan-2% microparticle began to alter colour on the 14th day. Throughout the observation period, the fade colour became intense and turns more yellowish, and the red colour at the chitosan-2% microparticle became impaired.

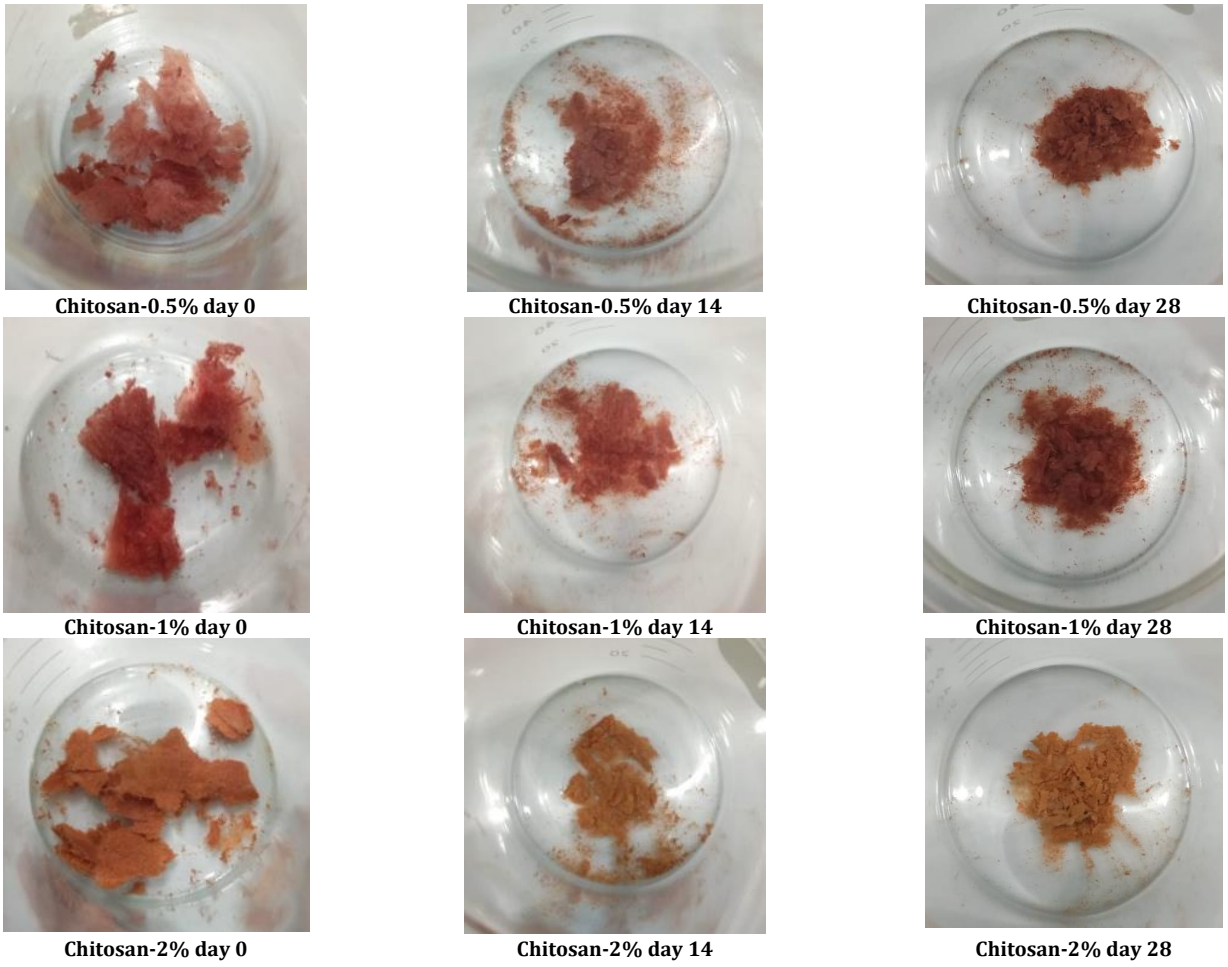

Fig. 4: Colour changed of chitosan microparticles containing beetroot extract using various concentration of chitosan matrix at day 0, 14 and 28. The microparticles was kept in climatic chamber at condition 40±2 °C and relative humidity 75±5%. The pictures were taken without magnification

The chitosan-1% microparticle performed the best among the microparticle samples mentioned in terms of colour retention over the course of the 28 d observation period. This result also related to level of betanin inside microparticle, which revealed the lowest reduction of betanin during 28 d storage compared to other microparticle preparations. The chitosan-2% microparticle showed the greatest decrease in betanin levels indicated by a more yellowish-orange colour and the red colour's intensity in the microparticle likewise diminishes after 28 d of storage.

CONCLUSION

The active ingredient betanin in beetroot extract can be maintained to be more stable during storage by encapsulated in chitosan microparticles. The capacity of the active ingredient to be encapsulated and protected was affected by the concentration of chitosan used in the microparticles preparation. In comparison to chitosan-0.5% and chitosan-2%, chitosan-1% microparticle showed the highest level of encapsulation efficiency. Based on the evaluation betanin level and colour stability tests for 28 d, chitosan-1% microparticles outperformed 0.5% and 2% chitosan microparticles in terms of betanin stability.

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AUTHORS CONTRIBUTIONS

Anita Sukmawati drafted the article and worked on the overall research strategy while Clara Cendera Marthadilla and Isna Vira Risdiyanti contributed to the data gathering and writing research report.

CONFLICT OF INTERESTS

There are no competing interests in this research.

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