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Original Article

FORMULATION AND STABILITY EVALUATION OF GELS CONTAINING CHITOSAN MICROPARTICLE-LOADED BEETROOT (*BETA VULGARIS*, LINN) FOR TOPICAL SKIN BRIGHTENING

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ABSTRACT

Objective: The objective of this research is to investigate the effect of Hydroxypropyl Methylcellulose (HPMC) concentration as a gel base for chitosan microparticle-containing beetroot (*Beta vulgaris*, Linn) toward gels stability and skin brightening effect.

Methods: Ionic gelation was used to make microparticle using chitosan 1% solution and beetroot dry extract as active component. Scanning Electron Microscope (SEM) and active substance loading were used for physical characterisation. The MP then was added to HPMC-based gels at 0.5, 1.0, and 1.5% *w/w*. Gels were tested for viscosity, pH, and active component stability. Gels were tested for skin lightening on humans.

Results: Results reveal beetroot extract may be loaded into chitosan microparticle with a Drug Loading (DL) of 23.27±0.057% *w/w*. HPMC gels had a pH of 5-5.4 and increased viscosity related with HPMC content. Gels showed colour instability after 6 cooling-heating cycles and decreased betanin levels on day 7 at 40±2 °C and RH 75±5%. HPMC 0.5% gel brightened human skin more than other HPMC gels.

Conclusion: The 0.5% HPMC gel base had the smallest betanin reduction during the accelerated stability test, compared to the 1.0 and 1.5% HPMC gels. The formulation of chitosan microparticle gel loading beetroot extract with 0.5% HPMC gel base had brightened skin better than the other two formulae.

Keywords: *Beta vulgaris*, Betanin, Chitosan, Microparticle, Skin brightening, Stability

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INTRODUCTION

Skin pigmentation can be induced by the presence of Reactive Oxygen Species (ROS) from Ultra Violet (UV) light due to the stimulation of melanin synthesis by melanocytes with exposure to UV light [1]. This condition, known as melanogenesis, leads to an excessive generation of skin pigments, resulting in the darkening of the skin, which is aesthetically undesirable. Hence, numerous studies have highlighted the significance of antioxidant properties in controlling skin pigmentation by mitigating the impact of reactive oxygen species (ROS) [2]. One of the plants containing antioxidant is beetroot (*Beta vulgaris*, Linn), is a plant that is rich in antioxidants. The primary contributors to the overall antioxidant capacity of beetroot are betalains and polyphenols [3]. Betalains are pigments that may dissolve in water. They consist of two sub-groups of compounds: purple-red betacyanins and yellow-orange betaxanthins, Betalains has antioxidant abilities and have been reported to have immunosuppressive, anti-inflammatory, hepatoprotective, and antitumor activity. Beetroot also contains many phenolic acids, including hydroxycinnamic and hydroxybenzoic acids, as well as flavonoids. These phenolic compounds have been identified for antioxidants activity [4].

Phytochemicals derived from plants have the potential to offer a largely opportunity for the development of novel medications to be used in the cosmeceutical and pharmaceutical sectors. The utilization of plant-based bioactive compounds in the development of skin care products is consistently emphasized due to the potential adverse effects that synthetic active ingredients may present [2]. However, betalains is susceptible to degradation due to effect of pH, temperature, and light exposure [5]. Hence, it is required to develop a delivery system that can efficiently protect the stability of betalain for prolonged utilization using microparticle.

Microparticles provide the benefit of protecting and preserving active chemicals from potential harm caused by elements such as temperature, humidity, oxygen, and microorganisms [6]. To encapsulate microparticles, polymers are necessary as a matrix during the preparation process. In this study, chitosan was utilized as a matrix for encapsulating beetroot juice extract in this study. Chitosan possesses various benefits such as being a hydrophilic polymer, having excellent mucoadhesive characteristics, low toxicity, biodegradability, strong mechanical strength, and the ability to enhance penetration across mucosal barriers [7–11]. Previous studies also showed that the microparticles with chitosan matrix concentration of 1% *w/v* have a higher Encapsulation Efficiency (EE) and Drug Loading (DL) compared to concentration of matrix 0.5% and 2% *w/v,* respectively [12], therefore in this research microparticles were used with a chitosan matrix concentration of 1% *w/v*.

To facilitate the active substance delivery to the epidermis of the skin, the microparticle then formulated in a topical matrix formulation such as gels. Gels for dermatological product offer several benefits, including thixotropic flow, greaseless, simple spread ability, easy washing, emollient action, nonstaining, and compatible with various excipients [13]. Hydroxy Propyl Methyl Cellulose (HPMC) gel was selected in this research as it offers non-toxic and biodegradable molecules. In addition, it makes intra and inter-molecular as well as hydrophobic interactions due to the presence of polar (hydroxypropyl) and non-polar (methyl) group in HPMC [14]. Nevertheless, the viscosity of the gel formulation would affect the release of active substances through the targeted skin. Gels with higher viscosity showed slower drug release. On the other hand, the research by Binder *et al.* (2019) found that adding the gelling agents to the dermal preparations can improve skin penetration by increasing skin contact [15]. In this research, we explored the effect of various concentrations of HPMC gels to the physical properties stability of the gel containing beetroot-chitosan microparticle. Furthermore, the ability of the gel containing beetroot-loaded chitosan microparticle for skin brightening also evaluated.

MATERIALS AND METHODS

Materials

Microparticles were made from chitosan (MW 100-200 kDa, CV Chi Multiguna, Indonesia), sodium tripolyphosphate, acetic acid glacial, tween 80, betanine (Aldrich) and distilled water. Gels formula consist of HPMC (high viscosity), propylene glycol, methylparaben, and distilled water. Bratachem, Indonesia, provides all resources unless noted differently. The dried extract of beetroot (*Beta vulgaris*,

Linn) was prepared through freeze drying the beetroot juice in a solution containing 1% citric acid. The Laboratory of Biology, Faculty of Education, Universitas Muhammadiyah Surakarta, Indonesia, identified the beetroot (*Beta vulgaris*, Linn) with Certificate No: 093/A. E-1/lAB. BIO/III/2022.

Preparation of dried beetroot extract

The beetroot was prepared by cleaning, peeling, and cutting it into smaller pieces. Then, 400 g of the peeled beetroot was mashed in a blender together with 400 ml of a 1% citric acid solution. Subsequently, the beetroot juice was extracted from the pulp by employing a clean cotton cloth and subjected to a freeze-drying process for a duration of four days. The beetroot was processed into powder and stored in the refrigerator. The dry material then employed for the preparation of microparticles.

Preparation of beetroot-loaded chitosan microparticle

Chitosan microparticle containing beetroot dry extract (*Beta vulgaris* L) was produced using the ionic gelation process. A chitosan solution at a concentration of 1% *w/v* was used as a matrix. A 1% *w/v* solution of chitosan matrix was created by dissolving 3.75 g of chitosan in 375 ml of a 1% acetic acid solution for 2 h using a magnetic stirrer at a speed of 1000 rpm. Afterward, 1.875 g of beet root juice, previously dissolved in 3 ml of distilled water, was introduced into the chitosan matrix solution and continuously stirred at 600 rpm for 15 min. Tween 80 was introduced in an amount equivalent to 0.2% of the total volume while maintaining agitation at a rate of 600 rpm. A crosslinking agent, 30 ml of 1% *w/v* sodium tripolyphosphate then was gradually added to the solution using a syringe. The stirring process was maintained at a speed of 600 rpm for up to 3 h. The particle was subsequently separated by centrifugating the solution for a duration of 15 min, 3000 rpm. The resultant particle was afterward washed three times with 3 ml of distilled water. The moist particle was subjected to freeze-drying for a duration of three days, after which it was transferred to a container wrapped with aluminium foil and stored at a temperature of 4 ℃.

Characterization of beetroot chitosan in microparticle

Characterisation of chitosan microparticle include the morphology of particle and the loading of active substances in the microparticle. Microparticle shape characterization was carried out by observing the microparticles using a Scanning Electron Microscope (SEM). Dry microparticles were placed on a carbon disk, then coated with gold in a vacuum for 5 min, then put into a chamber and bombarded with electrons. The results were observed at a voltage of 15kV with a scale of 10 – 200 µm at a magnification of 5000 times.

The quantification of the active component loaded in the microparticles was determined as betanin, which is a red pigment belonging to the betacyanin group found in beetroot extract. To investigate the entrapment of betanin in chitosan microparticles, 100 mg of the microparticles were dissolved in 2 ml of 1% acetic acid. This solution was then mixed with 8 ml of distilled water in a 1:4 ratio. To separate the chitosan debris, the solution was subjected to centrifugation at a speed of 3000 rpm for 5 min. The absorbance of a clear solution was measured at 528 nm using a UV-Vis spectrophotometer (Genesys 10S) to determine the quantity of betanin in the sample. The amount of betanin was calculated using the calibration curve equation Y = 0.1930X-0.0037. Equations (1) and (2) were utilized to calculate the EE and DL of betanin within chitosan microparticle (MP).

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

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

Formulation of gels containing beetroot-loaded chitosan microparticle

In this research, 3 gel formulas were made with HPMC concentrations of 0.5, 1.0 and 1.5%, totalling 70 g of gel in each formula and loaded with 2.5% *w/w* of beetroot-chitosan microparticle. Gel preparations were made by expanding 0.35, 0.70 and 1.05 g of HPMC in 28 ml of distilled water at a temperature of 80–90 °C. In a separate container, 0.14 g of methylparaben was dissolved in 10.5 g of propylene glycol. Beetroot chitosan microparticles (1.75 g) were dispersed in the remaining distilled water. The microparticle dispersion was mixed into a solution of methylparaben and propylene glycol, then added gradually into the HPMC that had been developed previously, then stirred until the gels were well dispersed. The gels were then homogenized using a mixer for 6 min at 2000 rpm.

Evaluation of stability of the gels

Stability testing was carried out on organoleptic, pH, viscosity and betanin levels in the gel preparations. Physical stability testing was carried out using a cooling-heating cycle for 6 cycles. For each cycle, the gel was stored at 4 ℃ for 24 h, then continuing at 40 ℃ for 24 h. After each cycle, the gel's physical parameters were evaluated, including organoleptic (gel colour and texture), viscosity, and pH. Colour and texture evaluation was carried out by observing the appearance the gels using camera (OPPO A31 12MP). Gel viscosity was measured using an AMETEK Brookfield DV-1 Digital viscometer (spindle number 7) at a rotational speed of 50 rpm for 10 seconds and pH evaluation was carried out using the OHAUS Starter 3100 pH meter.

Evaluation of the stability of betanin levels in gel preparations was measured using an accelerated stabilization test by storing the gel preparations in a climatic chamber at a temperature and Relative Humidity (RH) of 40±2 ℃/75±5% for 7 days. Betanin levels in the gels were measured at day 1 and day 7 by weighing 1 g of gel for each formula and dissolving it in 10 ml of distilled water. The solution was then centrifuged for 10 min at 8000 rpm to separate the beetroot chitosan microparticles from the HPMC gel base. Afterward, the gathered microparticles were rinsed twice with 10 ml of distilled water to remove any residual HPMC gel. The microparticle was then dissolved in a 2 ml of 1% acetic acid to disintegrate the chitosan matrix. Following that, 8 ml of distilled water was introduced and vigorously agitated to extract the bioactive compound betanin. The solution was subsequently subjected to filtration, and the amount of betanin was evaluated using the same method as previously mentioned for EE and DL evaluations.

Evaluation of skin brightness

An investigation was conducted to assess the brightening impact of a gel formulation containing beetroot-loaded chitosan microparticles. The study was performed on human hand skin and was deemed ethically acceptable, as confirmed by Ethical Eligibility Letter No.4533/B.1/KEPK-FKUMS/X/2022. The research involved 9 respondents. Each HPMC gel preparation formula of 0.5%, 1.0%, and 1.5% containing beetroot-loaded chitosan microparticle was used by 3 respondents. The selection criteria for participants were women between the ages of 18 to 25 year, with no previous record of skin allergies and willing to participate as respondents. The experiment was conducted over a period of 4 w. The gel was applied to the back of the participant's left hand with the area of 7x5 cm. The right hand of the respondent was utilized as a control. During the testing period, both hands should not use other moisturizing or skin lightening products to ensure unbiased result. The gel is used 2 twice daily, in the morning and evening for 2 h. During testing, respondents were still able to carry out normal activities under sunlight. Observations were carried out once a week on the backs of both respondents' hands to see physical changes in the respondent's skin. Documentation of the observation was carried out by taking pictures using a camera (Oppo A74 without flash) in an open area without lighting.

RESULTS AND DISCUSSION

Preparation and characterisation of beetroot chitosan microparticle

The microparticle was prepared using the ionic gelation process. In this method, the polyelectrolytes undergo crosslinking when their multivalent ion pairs are present. Chitosan was dissolved in a solution of 1% acetic acid using a protonation reaction mechanism. The amine group absorbed the hydrogen ion (H^+) generated by acetic acid, resulting in its conversion into a positively charged form (NH_3^+). The cross-linking process occurs when the negatively charged member of the tripolyphosphate polyanion interacts with the positively charged amine group in a complex manner [16].

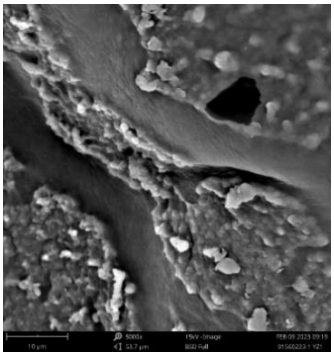

Fig. 1: The morphology of beetroot chitosan microparticle using 1% chitosan concentration as a matrix. The fig. was taken at 5000 times magnification

Fig. 1 showed the chitosan microparticles using 1% matrix concentration loaded with beetroot extract. The microparticle showed a non-spherical but rather flakes with an uneven and wrinkled surface. Previous research by Arianto, *et al.*, showed that the alginate matrix particles look spherical, but the chitosan, chitosan-alginate, and chitosan-alginate-calcium matrix particles were irregularly shaped and the surface of the chitosan-alginate and chitosan-alginate-calcium is smoother than the surface chitosan matrix [17]. Thus, it can be concluded that the use of chitosan as a microparticle matrix tends to produce non-spherical microparticle shapes and uneven surfaces since during the drying process the water adsorbed on the wet microparticles and pushed out. As a result, the microparticle structure becomes a non-spherical shape [18].

An assessment of the DL and EE was conducted to establish the data for determining the quantities of active substances in the microparticles during the stability test. Betanin was used as a standard for determine the active substances of beetroot extract. The betanin content within the chitosan microparticle was determined using DL, whereas the efficiency of betanin entrapment within the chitosan microparticle was assessed using EE. In this study, chitosan microparticles containing beet root juice produced DL of 23.27±0.057% *w/w* and EE of 9.32±0.023%.

Evaluation stability of the gels containing beetroot chitosan microparticle

The evaluation of colour and texture of the chitosan microparticle gel preparation of beetroot extract using HPMC concentrations of 0.5%, 1.0% and 1.5% showed that the gel preparations were red in colour with texture containing particles dispersed in the gel's preparation (fig. 2).

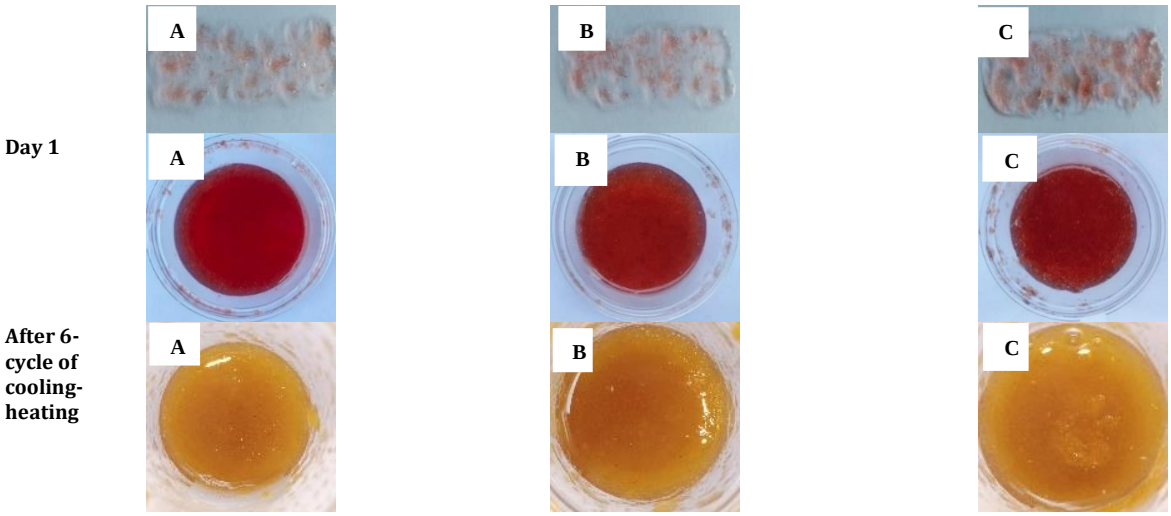

Fig. 2: Homogeneity and colour appearance of gels containing beetroot extract chitosan microparticle in 0.5% HPMC (A), 1% HPMC (B), and 1.5% HPMC (C) at day 1 and after 6-cycle of cooling-heating cycles

All the gels using various concentrations of HPMC had the same colour as they contain the same amount of microparticles. All gels containing chitosan microparticle experienced a colour change after 6 cycles to become yellowish. This colour change was occurred by the hydrolysis reaction in the N=C bonds due to a high temperatures, therefore, changing the betanin compound into betalamic acid (yellow) [19]. Variances in the concentration of the HPMC gel base did not have a significant effect on the colour change in the gel preparations (fig. 2A-C).

Rheological properties of the gels were evaluated by the viscosity stability. The evaluation of gel's viscosity indicated that the higher concentrations of HPMC lead to increased viscosity in the preparation. This phenomenon was also observed in the investigation carried out by Pan *et al.* [20]. The evaluation of the viscosity stability of the all the HPMC gels loaded with beetroot-chitosan microparticle exhibited consistent viscosity values after undergoing 6 cooling-heating cycles (fig. 3A). It can be concluded that HPMC concentration of 0.5%, 1.0%, or 1.5%, can be effectively maintained the viscosity stability of the gel preparation and ensuring its physical stability.

The pH values of the HPMC gels containing beetroot chitosan microparticle with concentrations of 0.5%, 1%, and 1.5%, were found in range between 5 and 5.4 (fig. 3B). This adheres to the pH range specifications for topical medicines, specifically based on the pH levels of the skin, which should be between 4.5 and 6.5 [20]. The pH of the preparation dropped in correlation with the increasing concentration of HPMC employed as the gel basis ($P<0.05$). This phenomenon diverges with the findings of prior research conducted by Sharon *et al.*, in which the pH of the preparation rose as the amount of HPMC in the gel preparation increased [20]. The pH levels after 6 cooling-heating cycles revealed a minor decline in pH values for all formulas compared to the condition before the cooling-heating cycling test (fig. 3B). However, this difference was not statistically significant ($P>0.05$). It can be inferred that the pH values remain stable throughout the stability testing process for all concentrations of HPMC gel base.

The amounts of betanin in gel preparations made from beetroot (*Beta vulgaris* L.) dry extract with different concentrations of HPMC gel base (0.5%, 1%, and 1.5%) were measured on days 1 and 7. The results indicated a decline in betanin concentrations in

all formulations (fig. 4). The ANOVA single-factor statistical test followed by the t-test, demonstrated that changes in the concentration of the HPMC gel base had a significant impact on the percentage decrease in betanin levels in the gel preparation ($P<0.05$). The 0.5% HPMC gel showed the least amount of decrease in betanin levels (table 1) and this difference was statistically significant when compared to the 1% and 1.5% HPMC gels ($P<0.05$).

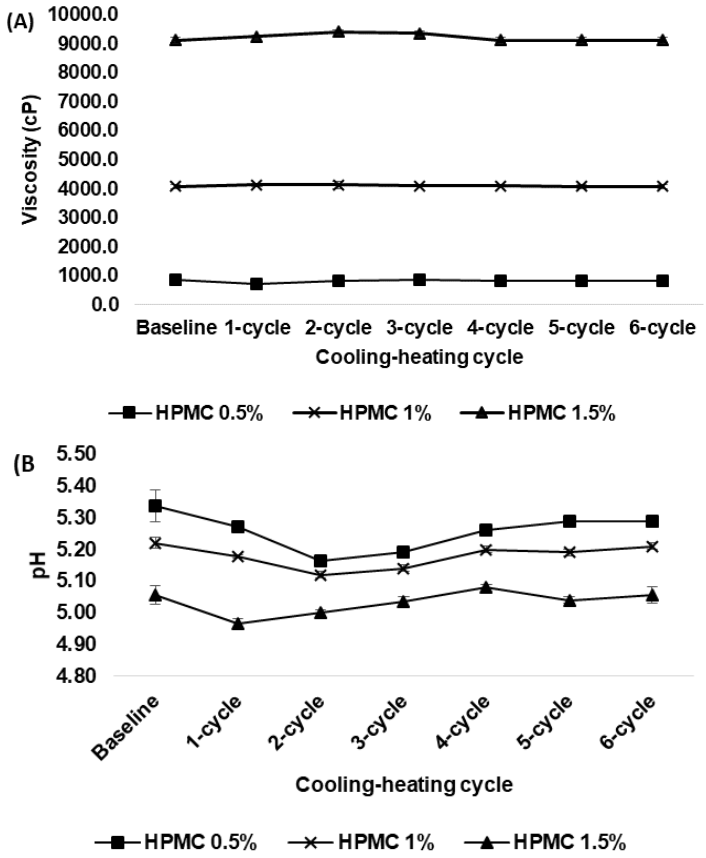

Fig. 3: Viscosity (A) and pH (B) of the HPMC gels (0.5, 1 and 1.5%) loaded with beetroot chitosan microparticle for 6-cycle of cooling-heating stability testing (n=3)

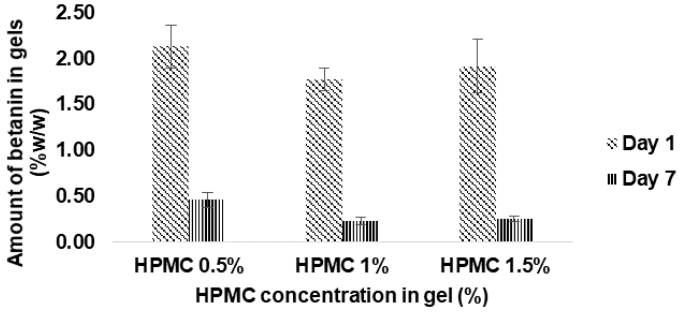

Fig. 4: Amount of betanin in HPMC gels with concentration of HPMC 0.5, 1 and 1.5%. The level of betanin in gels were evaluated at day 1 and day 7 (n=6)

Table 1: Average level of betanin and percentage decreasing of betanin levels in HPMC gels containing beetroot loaded chitosan microparticle at days 1 and 7 (n=6)

HPMC (%)	Amount of betanin in gels (% *w/w*)		Percentage decreasing level of betanin in gels (%)
	Day 1	Day 7	
0.5	2.14±0.234	0.46±0.084	78.24±4.319
1	1.77±0.127	0.23±0.037	86.74±2.906
1.5	1.92±0.287	0.25±0.032	86.68±1.669

Based on these findings, it can be inferred that the 0.5% HPMC gel possesses the capacity to maintain stable betanin levels, as it exhibits the lowest percentage decline in betanin levels. There was no statistical disparity in the capacity to maintain the betanin levels in gels for both HPMC 1% and 1.5%. The findings diverge from the prior study conducted by Chaerunisaa *et al.*, which shown that variations in HPMC concentrations in the gel formulation had no impact on the levels of atenolol during a 56-day storage period [21]. Castro-Enríquez *et al.* identified several aspects that contribute to the degradation of betalain compounds during encapsulation, including the type of matrix, the manufacturing process employed for encapsulation, and the porosity of the microencapsulation matrix [22]. The non-spherical and porous morphology of the microparticles as well as the inclusion of water components in the HPMC gel facilitated rapid release of the active material and accelerate the deterioration of betanin in microparticles [22, 23].

Skin brightness evaluation

The efficacy of the gels for skin brightening was evaluated by applying all formulas of the gels to the back of the respondent's left hand. The back of the right hand was utilized as a control without any gel application. The gel was applied for duration of 4 w. The results (fig. 5) were quantified in a score that represents the skin's capacity to enhance its brightness. A score of 1 denoted a skin-brightening effect, whereas a score of 0 denoted none. The outcomes of the assessment of skin brightening were presented in table 2.

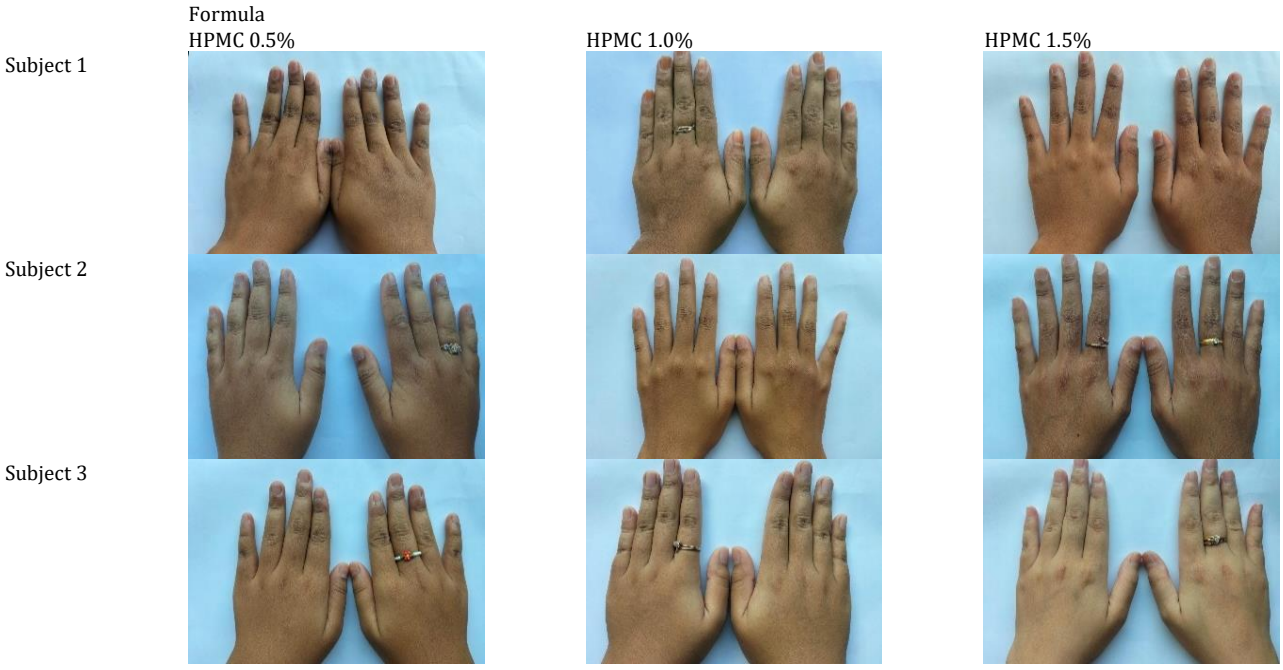

Fig. 5: Skin appearances after treated 4 w with HPMC gels containing beetroot loaded chitosan microparticle (left hand), while the right-hand side used as a control

Table 2: Brightening score of skin after 4 w treatment with HPMC gels containing beetroot loaded chitosan microparticle (n=3)

Subjek No.	Brightening score*		
	HPMC 0.5%	HPMC 1.0%	HPMC 1.5%
1	1	0	0
2	1	0	0
3	0	1	1
Total score	2	1	1

*Score 1 indicated a skin-brightening effect, while 0 indicated no skin-brightening effect.

The results indicated that HPMC 0.5% gel had brightening outcome for two subjects, while HPMC 1.0% and 2.0% gels only had the brightening outcomes for one subject each. It indicated that the gel containing beetroot-chitosan microparticles in 0.5% gel was more effective in brightening the skin compared to the 1.0% and 1.5% HPMC gels. The gel containing 0.5% HPMC was recognized for its greater capacity to preserve betanin throughout time (table 1). In addition, variations in the outcomes of skin brightening can be influenced by external factors such as the amount of sunshine exposure and environmental pollutants [24].

CONCLUSION

The concentration of the HPMC gel base in the range of 0.5-1.5% w/w did not affect the viscosity and pH of the gel's preparation. However, variations in the concentration of the HPMC gel base did impact the stability of the active ingredient in chitosan microparticles containing beetroot extract. The 0.5% HPMC gel base exhibited greater stability of the active component betanin, as seen by the smallest decrease in betanin levels throughout the accelerated stability test, in comparison to the 1.0 and 1.5% HPMC gels. The formulation of chitosan microparticle gel loading beetroot extract using 0.5% HPMC gel base exhibited superior skin brightening effects in comparison to the other two formulae. It is necessary to reevaluate the skin whitening impact of using gels containing chitosan microparticle loaded beetroot extract on a more extensive sample size of participants as well as evaluate the level of pigmen on the skin.

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AUTHORS CONTRIBUTIONS

Anita Sukmawati prepared the paper and designed the study approach, while Shinta J. Wahyuningrum and Maghfiratul L. Utami assisted in collecting data and drafting the research report.

CONFLICT OF INTERESTS

There are no competing interests in this researchs

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