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Original Article

ENHANCING CELL METABOLIC ACTIVITY USING MICROPARTICLES CONTAINING BEETROOT (*BETA VULGARIS,* LINN) EXTRACT

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ABSTRACT

Objective: The aim of this study was to assess the efficacy of delivering beetroot (*Beta vulgaris*, Linn) juice extract, which contains antioxidants, using Ethyl Cellulose (EC) and chitosan microparticles on cell metabolic activity.

Methods: The beetroot extract microparticles were produced by using EC and chitosan as a matrix. Different concentrations of the matrix were used in the primary solution during microparticles preparation. The physical characterisation was conducted, including Scanning Electron Microscope (SEM) and zeta potential. The entrapment of the active substance was measured using the Encapsulation Efficiency (EE) and Drug Loading (DL). The Vero cell was treated with EC and chitosan microparticles for 28 d and the cell metabolic activity was measured using resazurin assay.

Results: The findings indicated that the entrapment of beetroot extract in microparticles was affected by the concentration of EC and chitosan. The delivery of an antioxidant substance from beetroot extract resulted in an increase in cell metabolic activity indicated by cells proliferation from day 7 to day 21, in comparison to the control group.

Conclusion: The antioxidant as an active compound from beetroot juice extract were successfully delivered to the cell via the EC and chitosan microparticle indicated by impact on metabolic activity. The metabolic activity of the cell is influenced by the quantity of active substance contained within the microparticle and the type of polymer used as the microparticle matrix. The EC microparticle demonstrated a greater capacity to stimulate cell metabolic activity in comparison to chitosan microparticles.

Keywords: Antioxidant, *Beta vulgaris*, Linn, Chitosan, Ethylcellulose, Microparticle, Metabolic activity, Proliferation

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INTRODUCTION

The high level of antioxidants in beetroot (*Beta vulgaris* Linn) is attributed from betalain, flavonoids, polyphenols, ascorbic acid, and inorganic nitrate compounds [1]. The primary source which are responsible for the purplish red colour of beetroot is betanin, the key component with a high antioxidant [2, 3]. In general, antioxidants are highly helpful in preventing oxidative stress, which is involved in the processes of a number of diseases, including cardiovascular disease [4]. Moreover, antioxidants reduce skin damage produced by free radical compounds by neutralizing oxygen molecules, eliminating free radical compounds, inhibiting reactive oxygen species (ROS), inhibiting lipid peroxidase, and employing other techniques [5]. However, the active component of beetroot has the drawback of being unstable in the presence of heat and light. Microparticle technology is employed to keep the preparation stable, produced controlled release profile as well as maintained the therapeutics effectiveness [6]. Research by Dong and Wang showed that chitosan microparticles can regulate the release of active substances and preserve the stability of vitamin C, among other studies pertaining to the use of microparticles to retain active substances [7]. Sukmawati *et al.* have also conducted research utilizing an Ethyl Cellulose (EC) matrix to preserve the stability of betanin from beetroot juice. According to the findings, beetroot juice's microparticles 28 d storage had a lesser drop in betanin levels than beetroot juice that was not encapsulated with EC [8].

Particle properties, such as size and drug release profile, can be influenced by the concentration of polymer utilized in the microparticle matrix [9]. In this research, EC and chitosan were used as matrices for microparticles containing beetroot juice extract. Food and Drug Administration (FDA) approved EC for applied in biomedical applications due to its biocompatibility [10]. It is also believed that EC, a polymer resistant to temperature fluctuations [11] as a consequence, it will help the active ingredients enclosed in it remain stable. On the other hand, chitosan has been shown to exhibit outstanding mucoadhesive, biodegradable, and biocompatibility qualities when used to make microparticles [12, 13]. The research by Suzery *et al.* (2016) carried out additional study on phycocyanin encapsulation using a chitosan matrix. The phycocyanin encapsulation that is the subject of this study was expected to preserve its stability. The findings indicated that at high ambient temperatures, phycocyanin encapsulation with a higher chitosan matrix content had a more stable state [14]. Prior studies have demonstrated that beetroot extract microparticles prepared using EC and chitosan as a matrix exhibited antioxidant activity, with half-maximal Effective Concentration (EC_{50}) values ranging from 3 to 20 mg/ml depending on the type and concentration of matrix utilized [8, 15].

It has been demonstrated that antioxidants can both counteract free radicals and promote stem cell differentiation as well as stimulate cell proliferation [1, 16]. It was reported on research by Al-Harby *et al.* that the extract from *Beta vulgaris rubra* l was evaluated on Human Umbilical Vein Endothelial Cells (HUVEC). According to the study's findings, *Beta vulgaris rubra* extract has anti-aging, tissue-repair, and cell-regeneration properties since it can decrease inflammatory factors and promote the formation of vascular cells [1]. The other studies on the impact of antioxidants on intestinal goblet cells, as conducted by Akter *et al.* It indicated that the administration of *Terminalia ferdinandiana* extract can decrease cell viability in situations when the cells utilized are cancer cell models [17]. Hence, it is crucial to evaluate the antioxidant efficacy and transport of beetroot juice from microparticles for cellular metabolic function. This study would define how antioxidant from beetroot juice microparticle affect cell proliferation and evaluate the effect of the matrices of microparticles on the release of antioxidant in cells.

MATERIALS AND METHODS

Materials

The beetroot (*Beta vulgaris* Linn) was determined in the Biology Laboratory at the Faculty of Education, Universitas Muhammadiyah Surakarta (Certificate no. 093/A. EI/lab. Bio/III/2022) after being purchased from the local market in Sragen district, Central Java, Indonesia. Citric acid, EC, Polyvinyl Alcohol (PVA), Dichloromethane

(DCM), chitosan, acetic acid glacial, Tween 80, sodium tripolyphosphate (NaTPP), methanol, and distilled water were in pharmaceutical grade. Betanin was used as a standard for calibration curve. All the chemical in this research were obtained from Sigma Aldrich except for chitosan (CV ChiMultiguna, Indonesia). For the cell metabolic experiment, a Vero cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM). The cell metabolic assay was evaluated using Elisa reader after treated with resazurin (Sigma Aldrich) in phosphate buffer saline (PBS).

Preparation of beetroot juice's extract

A 200-gram beetroot was well washed and peeled before being diced and finely blended with 200 mililiters (ml) of a 1% citric acid solution using a blender. Once the pulp was extracted using a clean cotton cloth, the beetroot juice underwent freeze-drying for a duration of four days. Once this process was finished, beetroot powder was obtained and stored in the refrigerator until the microparticle preparation step.

Microparticle preparation

Microparticles were made using 2 types of matrices, namely EC and chitosan. The EC microparticle was prepared by varying the concentration of EC as a matrix with a concentration of 5-20% *w/v* in organic phase using the emulsification method. A 500 mg of EC was dissolved in 10, 5 and 2.5 ml of DCM using ultra-turrax to obtain EC solutions with concentrations of 5, 10 and 20% *w/v*, respectively. A 250 mg of beetroot powder was dissolved in 1 ml of distilled water and mixed in the EC solution until homogeneous by stirring at 16000 rpm for 3 min. The mixture was then dispersed in 50 ml of 0.5% PVA solution and continued with stirring using ultra-turrax (16000 rpm) for 3 min until an emulsion formed. The formation of microparticles was carried out by evaporating the DCM under a fume hood for 24 h by stirring continuously with a magnetic stirrer at a speed of 600 rpm.

The microparticles of beetroot juice with a chitosan matrix were produced using ionic gelation method. Three chitosan concentrations-0.5%, 1%, and 2% *w/v* in 1% acetic acid—were used to create the chitosan matrix solution for microparticles by dissolving 1.25 g of chitosan in 250 ml, 125 ml, and 62.5 ml, respectively. The stirring was done for three hours, with the first hour being completed at a speed of 1100 rpm and the latter two hours at a speed of 350 rpm. Each concentration of chitosan solution was combined with 0.625 g of beetroot juice that had been dissolved in 1 ml of distilled water. The mixture was then swirled for 15 min at 350 rpm using a magnetic stirrer. As much as 0.2% of Tween 80 then was added to a mixture of chitosan solution and beetroot extract. The mixture then agitated for 15 min at 350 rpm using a magnetic stirrer. Ten millilitres (mL) of the crosslinking agent 1% *w/v* Na TPP were applied dropwise to the mixture and continue to stir for 4 h at 350 rpm. The microparticle was separated by centrifugation for 15 min at 3000 rpm [18].

All the formed microparticles in each formula were washed using 5 ml of distilled water three times to remove the unencapsulated beetroot extract. The washed microparticles then dispersed in 3 ml of distilled water and dried using a freeze dryer. The dried microparticles were stored in the refrigerator in a dark glass container.

Characterization and evaluation of active substance entrapment in EC and chitosan microparticles

The physical properties of the EC and chitosan microparticles were analysed by examining their shape and size using a scanning electron microscope (SEM), as well as by measuring their zeta potential. The particle morphology was seen using a Scanning Electron Microscope (SEM Jeol J5M T300). Pictures were taken on the microparticles that had been coated with gold under vacuum conditions. The microparticle images were subsequently examined for their particle size utilizing the ImageJ software. The zeta potential of microparticles was determined by measuring the electrical potential of dispersed particles in a 10 ml solution of distilled water using the Horiba Scientific SZ 100 Particle Size Analyzer (PSA).

The entrapment of active substance of beetroot juice's extract was determined using betanin as a standard using direct method for both EC and chitosan microparticles. A 50 mg of EC microparticles containing beetroot juice's extract were dissolved in 1 ml of DCM, then 4 ml of distilled water was added to extract the beetroot extract and shaken. The aqueous phase on the top was filtered and taken. While for chitosan MP, the 50 mg of chitosan matrix, 50 mg of chitosan microparticles containing beet root juice were dissolved in 1 ml of 1% acetic acid. The active component betanin then was dissolved in the solution by adding a total of 4 ml of water. After that, the insoluble components were separated from the mixture by centrifuging it for five minutes at 3000 rpm. The clear solution from EC and chitosan MP was taken then the absorbance was measured using a Spectrophotometer UV-Vis (Genesis 10S) at 532 nm. The encapsulated active substance's content was determined by calibrating the calibration curve Y= 0.2055x-0.0325 using betanin as the standard. Equations 1 and 2 were used to determine the Drug Loading (DL) and Encapsulation Efficiency (EE) of betanin in microparticles.

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

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

Evaluation of EC and chitosan microparticles on cell metabolic activity

The impact of EC and chitosan MPs, which include an extract from beetroot juice, on the metabolic activity of cells was assessed by measuring the reduction of resazurin upon entry into live cells [19]. The Vero cell line was employed to evaluate the efficacy of microparticles containing beetroot juice. The cells were grown in 96-well plates with a density of 10,000 cells per well using DMEM as a cell medium. The cells were subsequently cultured for a duration of 24 h. Following a 24-hour period, 100 µl microparticles suspension (10 mg/ml) containing beetroot juice extract were cultivated with cells, using various matrix compositions (EC 5%, 10%, 20%, and chitosan 0.5%, 1%, and 2%). The cell media was replaced with fresh media every other day. Cell viability was evaluated at 7 d intervals over a period of 28 d. The cellular metabolic activity at each time point was assessed by adding 20 µl of resazurin solution in PBS (0.15 mg/ml) into each well-containing cells and microparticles. The plate was then placed in an incubator for a duration of 4 h. The Elisa Reader was used to measure the intensity of resorufin, which is a reduction product of resazurin, at a wavelength of 600 nm.

RESULTS AND DISCUSSION

Microparticle preparation using EC and chitosan as matrix

The emulsification process was utilized to create microparticles containing beetroot extract based on the characteristics of the EC and the extract from the beetroot juice. The beetroot extract was soluble in water, but the EC has a high solubility in organic solvents.

In this study, PVA was used as an emulsifier. The yield of microparticles produced from EC as a matrix with concentrations of 5, 10 and 20% *w/v* ranged from 32-40% where the amount of particle produced increases related to the increasing the EC concentration in primary emulsion during MP preparation. These findings support earlier studies that found that raising the drug-to-EC polymer ratio raised yield of MP. An increase in the rate of solvent diffusion from concentrated solution to emulsion was lead to the rise in yield of high EC polymers [20].

The increasing yield of microparticle related to increasing of matrix concentrations during MP preparation also found in chitosan MP containing beetroot juice's extract, although the different method was applied during MP production. The yield of chitosan MP prepared using ionic gelation method using chitosan concentration of 0.5%, 1 % and 2% *w/v* were varied from 3.5 to 28 %, whereas the yield was higher related to increasing of chitosan concentrations during MP preparation [21]. The dense structure of chitosan in high-concentration solutions helps to limit the occurrence of particle loss in the formation and collection process, which is why the yield of microparticles increases with increasing concentration of the chitosan solution used in microparticle preparation [15].

Physical characterisation and entrapment of active substances in EC and chitosan microparticles

The SEM results indicated that the particles produce from EC matrix had a non-spherical shape and contained several cavities. These cavities were formed due to the rapid evaporation of organic solvents from the microparticles (fig. 1a-c). Particle size analysis conducted using ImageJ indicated that the concentration of the EC polymer during microparticle production directly influenced the size of the particles, with higher concentrations resulting in larger particles (table 1). Increasing the viscosity of the organic phase during microparticle preparation leads to the formation of a more viscous solution and inhibited the formation of small droplets. As a result, larger oil droplets were generated, leading to larger particle sizes [22, 23].

The SEM images of chitosan microparticles reveals that their morphology is non-spherical, resembling flakes with a wavy surface (fig. 1d-f). Previous research produced comparable findings when used NaTPP as a cross-linking agent [24, 25]. The particle diameter data indicated that increasing the chitosan concentration from 0.5% to 1% leads to an enlargement in size. However, at a concentration of 2%, there is a reduction in particle size (table 1). This result was on contrary to Rodriguez's *et al.* research findings, which observed that the increase in particle size corresponds with the increase in chitosan concentration during the microparticle manufacturing process [25]. Increasing the viscosity of the chitosan 2% matrix solution during manufacture can influence the tighter particle structure, leading to smaller particle size.

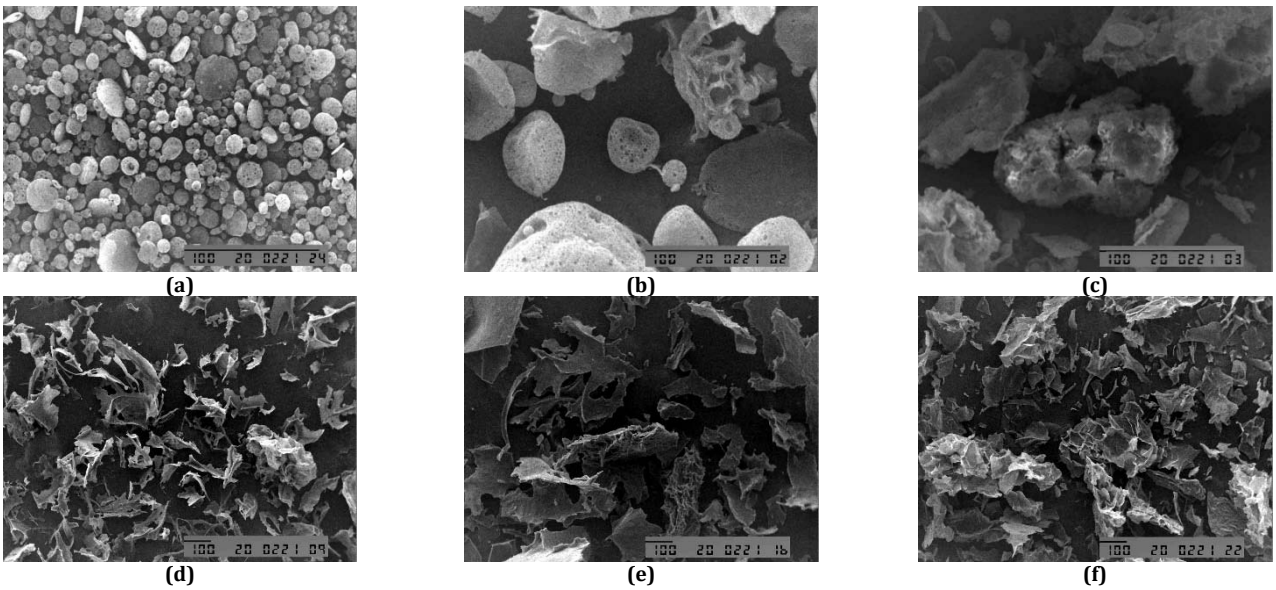

Fig. 1: Microparticles containing beetroot extract using concentration of EC 5% (a), 10% (b), 20% (c) and chitosan 0.5% (d), 1% (e), and 2% (f)

Table 1: The average particle size, the range of particle diameter and zeta potential of microparticle containing beetroot extract using ethyl cellulose (EC) 5%, 10% and 20% and chitosan 0.5%, 10% and 20% (n=3)*

Matrix concentration	Average particle diameter (µm)	Range of particle diameter (µm)	Zeta potential (mV)
EC 5%	5.1±0.61	4.1-6.1	(-)32.4±1.64
EC 10%	31.4±7.34	18.2-43.2	(-)8.1±0.6
EC 20%	40.9±10.64	20.6-58.6	(-)26.8±1.0
Chitosan 0.5%	105.8±46.06	86-140	(+)22.0±0.35
Chitosan 1%	110.9±96.03	86-135	(+)22.0±2.66
Chitosan 2 %	48.9±62.71	20-78	(+)9.2±1.88

The zeta potential of the microparticles was evaluated to assess the stability of the microparticles in the dispersion system. Particles with a zeta potential value of ±30 mV are regarded as being more stable in a dispersion system [26]. The microparticle, containing 5% EC, has a zeta potential exceeding 30 mV, indicating enhanced stability within the dispersion system. The microparticles exhibited a greater zeta potential when utilizing a 5% EC concentration, which was attributed to their smaller particle size compared to EC values of 10% and 20% [8]. In contrast to EC microparticles, this investigation revealed that all chitosan microparticles with concentrations of 0.5%, 1%, and 2% had a positive zeta potential. The positive zeta potential value of chitosan microparticles is a result of the presence of free ammonium groups in the chitosan polymer chain, as previously reported by Taurina *et al.* [27]. It may be verified that the chitosan matrix is located on the outer surface of the particle (table 1).

The encapsulation efficiency (EE) and drug loading (DL) were used to quantify the trapping of the active ingredient of beetroot juice's extract in the EC and chitosan microparticles. The EE value quantifies the efficiency of absorbing the active component into the microparticles, whereas the DL value measures the concentration of the active ingredient (betanin) in 100 g of microparticles. The results showed that the entrapment of beetroot extract in the EC microparticles improved by increasing of EC polymer concentration from 5% to 10% *w/v*, however the entrapment was reduced when the EC concentration increased to 20% *w/v* (table 2). The EC 20% *w/v* microparticles exhibit the greatest viscosity compared to other EC microparticles. As a result, the diffusion rate of the solvent, DCM, into an emulsion was reduced, and the microparticles form a densely fused and porous polymer layer. Consequently, the absorption of the active substance was diminished [20]. This result was in line with the research conducted by Rostinawati *et al.* (2023) using casticin-loaded EC microparticles [28].

Drug loading (DL) and EE values from EC matrix concentrations of 5, 10, and 15% *w/v* were subjected to a one-way ANOVA statistical test. The results indicated that DL and EE values are influenced by variations in matrix concentration ($p<0.05$). Increasing the EC concentration from 5% to 10% led to a significant increase in the DL and EE values of the microparticles. Similarly, altering the matrix concentration from 10% to 20% also resulted in a significant decreased in the DL and EE values of the microparticles ($p<0.05$).

Table 2: The Entrapment efficiency (EE) and drug loading (DL) of microparticle-containing Beetroot extract using ethyl cellulose (EC) 5%, 10% and 20% and chitosan 0.5%, 10% and 20% (n=3)*

Matrix concentration	EE (%)*±SD	DL (%*w/w*)* ±SD
EC 5%	5.9±0.06	11.8±0,11
EC 10%	7.5±0.12*	15.0±0,24*
EC 20%	6.4±0.11	12.7±0,22
Chitosan 0.5%	10.7±0.07	21.3±0.15
Chitosan 1%	16.3±0.10*	32.7±0.20
Chitosan 02 %	11.9±0.04	23.8±0.09

*Amount of active substance entrapped in microparticle calculated as betanin and represent as mean±SD (n=3), *Significant compared to the microparticle in the same matrix.

In chitosan microparticle, the DL and EE of MP indicated that the drug loading and encapsulation effectiveness of chitosan matrix microparticles are maximum at a concentration of 1% *w/v*, followed by a chitosan concentration of 2 % *w/v*, and lowest at a chitosan matrix concentration of 0.5% *w/v* (table 2). The findings of this study contradict the earlier research conducted by Suzery *et al.*, (2016), which demonstrated a positive correlation between concentration and encapsulation effectiveness [29].

The t-test statistical analysis results indicated that beetroot juice absorption capacity in the chitosan matrix can be enhanced by raising the chitosan matrix solution's content from 0.5% to 1% ($p<0.05$). An elevated chitosan concentration in the production of microparticles can enhance the capacity of chitosan to bind the active substances. Since the addition of Na TPP induced the development of cross-linking in the chitosan matrix, increasing the concentration of chitosan in the microparticles could increase its ability to bind the active components [30]. However, in this study also discovered that microparticles created using a 2% w/v chitosan solution concentration exhibited a reduction in DL and EE values in comparison to 1% chitosan microparticles, as indicated by a t-test result ($p<0.05$). This occurs due to a significant rise in the viscosity of the chitosan solution, which subsequently leads to an increase in the density of the chitosan matrix. Consequently, the ability of chitosan to absorb active substances is reduced [31]. Hence, the amount of active ingredient that can be encapsulated within microparticles created using a 2% w/v chitosan solution concentration was lower compared to microparticles formed from a solution concentration of 1% w/v chitosan.

When comparing the EE of microparticles utilizing both EC and chitosan matrices, it was shown that the chitosan matrix had a significantly greater EE, approximately double in value. The results suggested a positive interaction between the chitosan matrix and the aqueous beetroot juice extract, likely due to the hydrophilic characteristics of chitosan. The correlation between chitosan and aqueous extract was dependent on the concentration of the extract added until it reached a point of saturation where it can interact with the chitosan [32].

Effect of EC and chitosan microparticles on cell metabolic activity

An *in vitro* evaluation was conducted to assess the efficacy of delivering antioxidants from beetroot juice using microparticles on the metabolic activity of Vero cells. Cell growth was assessed by resazurin assay at a 7 d interval. The transition of the resazurin solution's colour from blue to pink indicated that the proliferation of Vero cells that subjected to beetroot microparticles. This change occurred due to the reduction of resazurin to resorufin by mitochondrial enzymes when it entered the viable cell [33].

The cell viability intensity, when exposed to EC beetroot microparticles at concentrations of 5%, 10%, and 20% *w/v*, showed a rise from day 0 to day 14 comparing to the control group (fig. 2a). The cells that were treated to EC beetroot microparticles with a 10% concentration showed the greatest level of cell metabolic activity, measured at 1.7±0.05 in its intensity. This finding aligns with the DL and EE tests conducted in this study, where the highest EE and DL was found in EC 10% microparticle-containing beetroot. Nevertheless, there was a slight decline in outcomes observed when employing beetroot microparticles with an EC concentration of 20%, which might be attributed to the reduced betanin content in the EC 20% microparticles in comparison to those with an EC concentration of 10%. Beetroot has a substantial amount of betanin, which exhibits potent antioxidant properties. Prior studies have demonstrated a positive correlation between the concentration of antioxidant-rich active component extracts and cell viability [34]. Zago´rska-Dziok *et al.* also conducted research that demonstrates a similar link between levels of extract and both antioxidant activity and cell viability [35].

The impact of different concentrations of EC in beetroot microparticles on cell viability indicated that there was a significant influence of variations in EC matrix concentration in microparticles on Vero cell growth for 28 d (ANOVA, $p<0.05$), particularly in cell treated with EC 10% microparticle containing beetroot extract.

Additionally, a 28 d assessment of Vero cell viability was also conducted in beetroot microparticles from chitosan matrix. The findings demonstrated significant progress in cellular proliferation. The proliferation of Vero cells exhibited an initial increase from day 0 to day 21, followed by a subsequent drop on day 28 (fig. 2b). Once cells have reached a state of confluence, their growth ceases or they initiate cell death, thereby requiring subculture. The presence of confluent cells, an increase in cell number, overfilling of the wells in the 96-well plate, lack of subculture, and unchanged media volume can lead to a decrease in cell viability. The maximum cell viability intensity on days 0, 7, 14, 21, and 28 was observed in beetroot extract microparticles with a chitosan matrix concentration of 2.0% *w/v*. This was followed by a chitosan concentration of 1.0% *w/v* and then chitosan 0.5% *w/v*. This finding contradicts the results of the EE, which indicated that the chitosan concentration of 1% *w/v* produced the highest EE value. Consequently, cell viability is anticipated to be enhanced when subjected to 1% MP chitosan.

The statistical tests using one-way ANOVA revealed that the intensity of Vero cell metabolic activity differed significantly between chitosan matrix in all concentrations ($P<0.05$) except for chitosan matrix of 1.0% *w/v* and 2.0% *w/v* ($P>0.05$). Increasing the concentration of the chitosan matrix from 0.5% *w/v* to 1% *w/v* had a notable impact on enhancing the viability of Vero cells. This is related to the EE at microparticle using chitosan concentrations of 0.5% and 1%.

In general, even though the chitosan microparticle contains a larger amount of the active component, it was shown that the microparticle with an EC matrix had a more significant impact on metabolic activity related to the capabilities to deliver antioxidant substance in beetroot extract. The EC matrix was found to have higher compatibility with Vero cells, despite its negative charge [8].

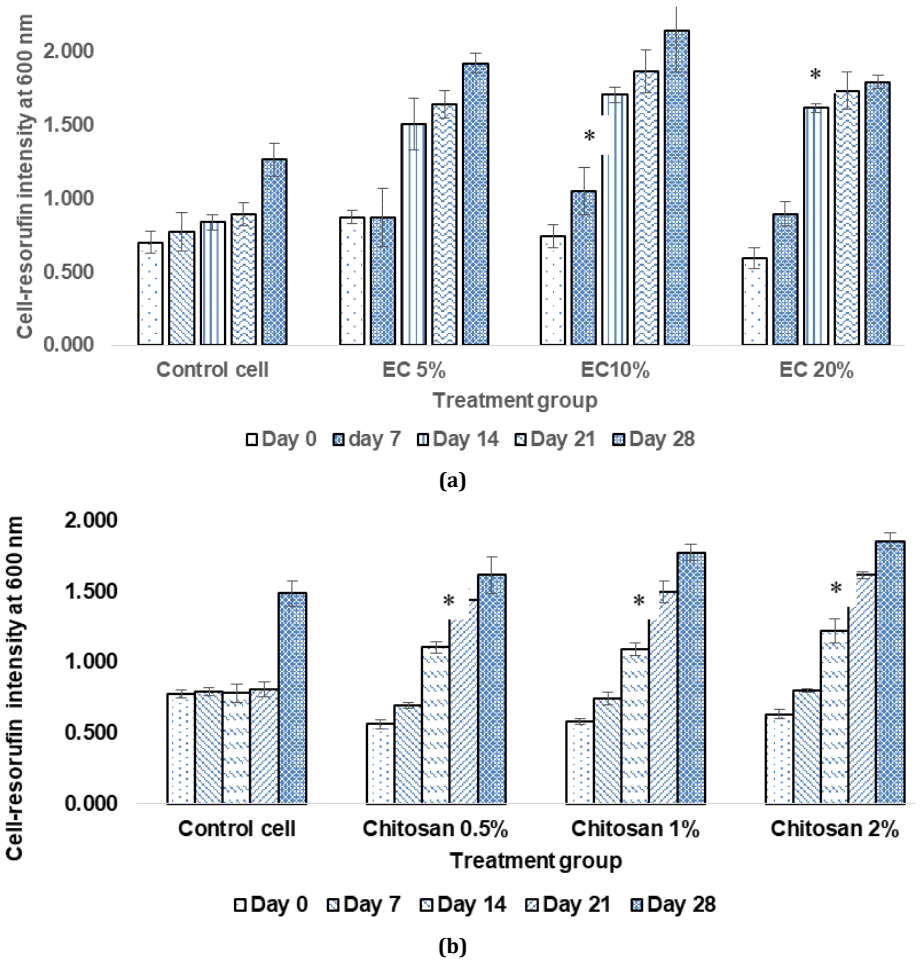

Fig. 2: Cellular metabolic activity was assessed using two types of microparticles: (a) ethyl cellulose (EC) and (b) chitosan, which containing beetroot juice extract. The metabolic activity was assessed using resazurin and quantified by measuring the intensity at a wavelength of 600 nm. (*) showed the significant value compared to control cell. The experiment was conducted with a sample size of 4 (n=4)

CONCLUSION

EC and chitosan were used to effectively load a beetroot juice extract into a microparticle system. The entrapment effectiveness of betanin, the active ingredient, was dependent on the concentration of EC or chitosan in the primary solution used for microparticle production. The assessment of antioxidant administration to Vero cells demonstrated a positive effect on cellular metabolic activity in comparison to the control cells. The metabolic activity of the cell depends on both the amount of active substance trapped within the microparticle and the kind of polymer used as the microparticle matrix.

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AUTHORS CONTRIBUTIONS

Anita Sukmawati made contributions to the overall research design and wrote the manuscript. Setyo Nurwaini contributed by supervising laboratory activity and supporting the writing of the paper. Jihan Naufa Hazimah and Anisa Jevi Romadani Saputri contributed to data collecting and the preparation of the research report.

CONFLICT OF INTERESTS

There is no conflict of interest

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