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Original Article

NANOENCAPSULATION OF CHITOSAN-ETHANOL EXTRACT OF RED BULB LEAVES (*ELEUTHERINE AMERICANA* MERR.) USING THE IONIC GELATION METHOD

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ABSTRACT

Objective: The red bulb is an endemic plant of West Kalimantan with high antioxidant activity. However, it is precarious and easily damaged.

Methods: This research aims to formulate red bulb leaf extract into nanoparticles to protect it from degradation. The polymer used is chitosan with Na-TPP as a cross-linker. The study aims to determine the concentration of chitosan that can form red bulb leaf extract nanoparticles and to characterize the resulting particles. The nanoparticles were prepared using the ionic gelation method by mixing Na-TPP, extract, and chitosan (1:1:6) with a magnetic stirrer at 1500 rpm for 5 h. The nanoencapsulation of the ethanol extract of red bulb was evaluated for characteristics including particle size distribution, polydispersity index, zeta potential, particle morphology, and entrapment efficiency.

Results: The nanoparticle formulations were characterized, yielding particle sizes for F1, F2, and F3 of 265.1 nm, 271.7 nm, and 299.8 nm, respectively, with polydispersity index values of 0.177, 0.208, and 0.194, respectively. The zeta potential values obtained in this study for F1, F2, and F3 were 1.10 mV, 0.43 mV, and 0.31 mV, respectively. The percentage inhibition of the free nanoparticle extract for F1, F2, and F3 was 22.328%, 17.853%, and 15.768%, respectively. The % inhibition value of the free extract against DPPH from the research results was 22.328±0.794% for formula 1, 17.853±1.048% for formula 2, and 15.768±0.780% for formula 3. The formulation that produced the best characterization results was F3, with a particle size of 299.8 nm, a polydispersity index of 0.194, and a zeta potential of 0.31 mV, although the particle morphology was less spherical.

Conclusion: The formulation that produced the best characterization results was F3, with a particle size of 299.8 nm, a polydispersity index of 0.194, and a zeta potential of 0.31 mV, although the particle morphology was less spherical. The % inhibition value of the free extract against DPPH from the research results was 15.768±0.780% for formula 3.

Keywords: Nanoencapsulation, Chitosan, Sodium tripolyphosphate, Ionic gelation, Red bulb ethanol extract

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INTRODUCTION

The red bulb (*Eleutherine americana Merr.*) is one of many plants with potential as a natural medicinal ingredient that can be developed. The red bulb, also known as Bawang Dayak, belongs to the Iridaceae family and is widely used by the people of West Kalimantan as a medicinal plant. The red bulb offers numerous benefits, including anti-inflammatory properties, hemostatic effects (stopping bleeding), and antitumor activity [1, 2]. Red bulbs contain vital compounds such as polyphenols, tannins, alkaloids, saponins, phenolics, and flavonoids [2-4].

The antioxidant activity of a 70% ethanol extract of the red bulb using the DPPH method has a high free radical-reducing effect, with an IC50 (Inhibitory Concentration at 50%) value of 31.97437 µg/ml [5]. The main characteristic of antioxidant compounds is their ability to capture free radicals [6]. Plants containing total phenolics correlate strongly with their antioxidant activity [7-11].

Plant extracts with various benefits are now being formulated into different dosage forms. A common challenge when developing an extract is the inconsistency in particle size and poor stability in the dosage form, which can lead to a decrease in the desired effectiveness of the formulation [12]. The same challenges will also be encountered when formulating the ethanol extract of the red bulb into a dosage form. Formulating the ethanol extract of the red bulb in the form of nanoencapsulation is one approach that can be taken to overcome these challenges.

Nanoencapsulation is a method of coating materials using nano-sized polymers [13]. The nanoencapsulation of red bulb ethanol extract can be achieved using the ionic gelation method with the polymer chitosan and the cross-linker Na-TPP. The basis of the ionic gelation method is the transformation of chitosan from a liquid to a gel due to ionic interactions with polyanions. This interaction occurs between the positively charged ammonium groups of chitosan and a negatively charged cross-linking agent [14].

Chitosan was chosen as the polymer for coating the nanoparticles of red bulb extract because it offers several advantages as a coating material for nano encapsulation. The advantages of chitosan include its versatility in physical forms (such as flakes, porous beads, gels, fibers, and membranes), biodegradability, ease of handling, and non-toxicity. Additionally, chitosan has heat-resistant properties, protecting the red bulb extract components from damage caused by high temperatures [15]. Chitosan will form ionic bonds with Na-TPP (sodium tripolyphosphate) as a cross-linker to create a "network" on the surface of the nanoparticles, which will stabilize the particles. The reason for using tripolyphosphate is its property as a multivalent anion that can form more stable cross-links with chitosan, which is non-toxic and has better membrane penetration characteristics [16]. Extracts formulated in the form of nanoencapsulation can achieve uniform particle sizes. The stability of the extract can also be improved due to the polymer, which protects the extract both physically and chemically. As a result, the antioxidant activity of the compounds contained in the extract can be preserved in the formulation [12].

Therefore, the nanoencapsulation of chitosan-red bulb ethanol extract (Eleutherine americana Merr.) was performed. Once the ethanol extract of the red bulb is formulated into nanoparticles, the resulting nanoparticles are expected to exhibit favorable characteristics.

MATERIALS AND METHODS

Materials

The materials used in this research were red bulb, 70% ethanol, concentrated H_2SO_4, Mg powder, concentrated HCl, acetic acid, distilled water, NaCl, gelatin, n-hexane, methanol, $FeCl_3$, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Lieberman-Burchad reagent, Dragendorff reagent, Wagner reagent, Mayer reagent, chitosan, and Na-TPP.

Instruments

The tools used in this research were a maceration vessel, blender (IllinQi fz-10), digital scales (Precisa Type X. B. 4200C), glassware

(Pyrex), rotary evaporator (Heidolph Type Heizbad Hal-VAP), water bath (Memmert Type WNB-14), crucible cup, hot plate (S. I. Analytics GmbH Type D-55122), electric oven (Modena), desiccator, pH meter, magnetic stirrer, centrifugator (P. L. C. Series), freeze dry (CHRIST® ALPHA 1-2 lDplus 101521), Particle Size Analyzer (Beckman Coulter type DelsaNano C), Zeta Potential (Beckman Coulter type DelsaNano C), Scanning Electron Microscopy (Hitachi TM 3000) and UV-Vis spectrophotometer (Reyleigh).

Plant determination

Red bulb leaves were collected from plantations in the Rasau Jaya sub-district, Kubu Raya district, West Kalimantan. The harvesting of Red bulb leaves is carried out during the dry season, in the afternoon when environmental conditions have improved, marked by a decrease in sunlight intensity, decreasing environmental temperature, slightly increasing air humidity, and the rate of plant evapotranspiration starting to decrease so that the plants begin to look fresh and green again. Red bulb leaves were determined at the Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Tanjungpura University, Pontianak, West Kalimantan with number 077/A/lB/FMIPA/UNTAN/2015.

Collecting and extraction of red bulb

The sample used was red bulb leaves from the Iridaceae family. The red bulb leaves were sourced from plantations in the Rasau Jaya sub-district, Kubu Raya district, West Kalimantan. The inclusion criteria for sampling were that the leeks should be harvested in the afternoon when they are 6 mo old, with leaves that are fully opened, green, and fresh. The exclusion criteria were that leaves should not be harvested in the morning and should not be less than 6 mo old or still curled, yellowish, or wilted.

The Red bulbs were collected, cleaned of dirt with clean water, and drained. The leaves were then chopped and dried in a drying cabinet at 40 °C. The dried leaves were stored in a dry container to avoid damage and contamination [5]. The extraction process was carried out on the dried leaves. Red bulbs were extracted using the maceration method. The sample, weighing 183.62 gs, was placed into a maceration vessel, and 70% ethanol was added until the sample was fully submerged. The container was then covered with aluminum foil. Maceration was conducted until the extraction became colorless, which took 8 d. The solvent was changed and stirred as often as possible every 24 h. The resulting ethanol extract was collected and filtered. The ethanol extract was then concentrated using a rotary evaporator at 40 °C until a concentrated extract of red bulb leaves was obtained. Finally, the extract was further concentrated by heating it in a water bath until a thick extract was achieved [17].

Drying shrinkage

The extract was weighed at 1 g and placed into a crucible previously heated at 105 °C for 30 min until a constant weight was achieved and tared. The extract was evenly spread in the crucible by shaking it until it formed a layer approximately 5-10 mm thick. It was then placed in an oven with the lid open, and the crucible lid was also dried at 105 °C until a constant weight was reached. The crucible must be closed immediately when the oven is opened. Place the crucible in a desiccator and allow it to cool. Drying is then continued at 105 °C until a constant weight is achieved, meaning that during two consecutive weighings, the weight difference should not exceed 0.50 mg per g of the substance used. The second weighing is performed after reheating for 1 hour [18, 19]. The remaining solvent in the extract can be calculated using the following formula:

$$\text{Drying Shrinkage} = \frac{A-B}{A} x100$$

Information:

A = Weight before heating

B = Weight after heating

Determination of ethanol soluble essence content

One g of the extract (W0) was macerated with 20 ml of 96% ethanol for 24 h in a bottle or container. The mixture was shaken periodically during the first 6 h, and left undisturbed for 18 h. The mixture was filtered promptly to avoid ethanol evaporation. The crucible containing the residue was then heated at 105 °C until a constant weight was reached (W1) [20].

$$\text{Ethanol Soluble Extract Content} = \frac{W1}{W0} \text{ x } 100\%$$

Description:

W0 = Initial weight of the extract

W1 = Constant weight of the crucible and sample after drying

Phytochemical screening

The phytochemical screening includes tests for alkaloids, flavonoids, saponins, steroids, triterpenoids, phenols, and tannins [21].

Making chitosan nanoparticles–red bulb leaf extract

1 ml of the red bulb leaves extract stock solution is mixed with 1 ml of 0.1% Na-TPP solution drop by drop, followed by a magnetic stirrer. Then, the Na-TPP and red bulb leaves ethanol extract mixture is gradually added to 6 ml of chitosan solution (with concentrations ranging from 0.1% to 0.3%) at room temperature, with stirring using a magnetic stirrer (at 1500 rpm for 5 h) until a nanoparticle suspension is formed [22].

Table 1: The Nanoencapsulation formula of chitosan-ethanol extract of red bulb-leaves

Material	Formula (% w/v)		
	F1	F2	F3
Ethanol extract from red bulb leaves	8	8	8
Chitosan (%)	0.1	0.2	0.3
NaTPP (%)	0.1	0.1	0.1

Description: F1 = Chitosan (0.1%); F2 = Chitosan (0.2%); F3= Chitosan (0.3%); Na-TPP = Natrium Tripolifosfat.

Nanoparticle characterization

Nanoparticle characterization included particle size, zeta potential, inhibition of the free extract nanoparticles against DPPH, and nanoparticle morphology [23]. Particle size was analyzed using a particle size analyzer (Beckman Coulter). A specific amount of the sample was placed into a cuvette and then into the P. S. A instrument. The parameters analyzed included the average particle diameter (Zave), polydispersity index (P. I), and zeta potential. Zeta potential was measured using Laser Doppler Electrophoresis (LDE) with Beckman Coulter equipment. Particle morphology was observed using scanning electron microscopy (Hitachi TM 3000). The nanoparticle powder of Red bulb leaf extract was coated with gold (Au) for 20 seconds. The observation was conducted at a resolution of 15 kV.

Verification of the spectrophotometric method with DPPH

A stock solution of DPPH was prepared at a concentration of 100 ppm by carefully weighing 5 mg of DPPH powder and dissolving it in methanol in a 50 ml volumetric flask up to the mark. The DPPH solution was then homogenized by gently swirling. A 30 ppm DPPH solution was made from the stock solution. The wavelength was scanned on the 30 ppm DPPH solution, measuring its absorbance from 450 to 550 nm to

determine the maximum wavelength. DPPH concentrations of 15, 20, 25, 30, 35, and 40 ppm were prepared by diluting the stock solution. The absorbance of these DPPH concentrations was measured using a UV-Vis spectrophotometer at the maximum wavelength of DPPH, which is 515.5 nm, to obtain absorbance values. Linearity was assessed from the linear regression of the standard curve between concentration and absorbance. The best regression equation is indicated by a correlation coefficient (r) of ≥ 0.999 [25].

The DPPH concentrations of 15, 20, 25, 30, 35, and 40 ppm were measured for absorbance at a wavelength of 515.5 nm using a UV-Vis spectrophotometer. Accuracy (precision) is the percentage recovery (% recovery). The percentage recovery is expressed using the equation:

$$\% \text{ Recovery Rate} = \frac{Ci}{Co} \times 100\ \%$$

Description: Ci = concentration obtained from the measurement

C0 = actual concentration

The DPPH concentration series of 15, 20, 25, 30, 35, and 40 ppm was measured for absorbance at a wavelength of 515.5 nm using a UV-Vis spectrophotometer. The measurements were replicated three times. Precision was assessed as the relative standard deviation (RSD). The equation expresses the RSD value:

$$RSD = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100\%$$

Percent inhibition of the free extract nanoparticles against DPPH

The inhibition measurement of free extract in nanoparticles was performed using the DPPH method, as used by Molyneux, with slight modifications. A 40 ppm DPPH solution was prepared by weighing 4 mg of DPPH, adding it to a 100 ml volumetric flask, filling it up to the mark with methanol, and then homogenizing it. The maximum wavelength (λ max) of the 40 ppm DPPH solution was determined using UV-Vis spectrophotometry, measuring its absorbance at a wavelength of 515.5 nm. The chitosan-ethanol extract of red bulb leaves nanoparticles suspension was centrifuged at 8000 rpm for 15 min to separate the free extract (filtrate) from the bound extract (residue) on chitosan and Na-TPP [22].

Next, the filtrate is collected and freeze-dried. The freeze-dried product is then dissolved using 1 ml of methanol and mixed with 2 ml of 40 ppm DPPH, followed by homogenization. The resulting solution is incubated at room temperature for 30 min, after which its absorbance is measured using a UV-Vis spectrophotometer. The inhibition of the free nanoparticle extract against DPPH is calculated using the following equation:

$$\text{Percentage inhibition} = \frac{\text{DPPH absorbance} - \text{absorbance of free extract}}{\text{DPPH absorbance}} \times 100\%$$

Data analysis

The data obtained from the characterization of the ethanol extract of red bulb leaf nanoparticles include particle size, polydispersity index, zeta potential, particle morphology, and the percentage inhibition of free extract in nanoparticles against DPPH. This data is analyzed descriptively in paragraph form and theoretically compared to provide an overview of the research results.

RESULTS AND DISCUSSION

Plant determination

The red bulb leaves was determined at the Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Tanjungpura University, Pontianak, West Kalimantan, with the number 077/A/lB/FMIPA/UNTAN/2015. The Red bulb plant samples submitted consisted of whole plants, including leaves, stems, bulbs, flowers, and roots. The determination results confirmed that the plant used in this study is indeed Red bulb, specifically *Eleutherine americana* Merr. from the Iridaceae family.

Collecting and extraction of red bulb

The red bulb plant was determined at the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Tanjungpura University, Pontianak. The collected raw materials were then subjected to wet sorting. The red bulb leaves, washed and drained for one night, are cut into small pieces approximately 1 cm long. After cutting, the leaves are dried in an oven at 40 °C until they are scorched and suitable for grinding. In this study, the drying process lasted for 3 d. This drying process aims to reduce the moisture content so that the sample is less prone to mold and bacterial growth, eliminate enzyme activity that could degrade the active compounds, and obtain a more durable sample during storage [28].

The samples, which have been dried into simplicia, are then dry-sorted to separate the simplicia from impurities obtained during the drying process. The dried red bulb leaf simplicia is powdered using a blender and then sieved with a 20-mesh sieve to obtain a homogeneous size. The resulting simplicia powder weighed 183.62 g from 1.00 kg of fresh red bulb leaves. Maceration was performed on 183.62 g of the red bulb leaves powder. The maceration process lasted 8 d using 7 l of 70% ethanol as the solvent. The extract was then concentrated into a thick extract weighing 47.2082 g.

Drying shrinkage

The purpose of the drying loss test is to standardize the extract to ensure that the extract used is standardized. Drying loss can determine the amount of solvent or other volatile compounds in the extract. The drying loss test is conducted using an oven at 105 °C until a constant weight is achieved. A continual weight means that the sample weights should not exceed 0.50 mg. The drying loss test conducted on the ethanol extract of red bulb leaves yielded 18.79±0.08% (w/w), which indicates that the extract falls within the range of a thick extract, precisely 5-30% [29].

Phytochemical screening

Phytochemical screening is used to qualitatively detect the presence of secondary metabolite compounds in plants that exhibit biological activity. Chemical compounds were identified in the extract using the color reagent method with a tube test. The results of the phytochemical screening showed that the red bulb leaf extract tested positive for flavonoids, steroids, and phenolics but tested negative for alkaloids, saponins, terpenoids, and tannins.

Determination of ethanol soluble essence content

Maceration was carried out on 183.62 g of powdered simplicia of red bulb leaf. The maceration process lasted 8 d, and 70% ethanol solvent was used for as much as 7 l. The extraction process was carried out in a glass container, and the solvent was replaced within 1x24 h. The liquid extract from the maceration that had been obtained was concentrated with a rotary evaporator to evaporate the solvent below its boiling point. A temperature of 40 °C was used to maintain the active compounds in the extract so that heating did not damage them. The crude extract obtained was then concentrated in a water bath so that the substances that were not needed would evaporate with the solvent. The thick extract obtained in this extraction process weighed 47.2082 g.

The yield value is the comparative value between the weight of the extract obtained and the weight of the simplicia powder used. The yield value obtained from the ethanol extract of red bulb leaves was 25.71% b/b.

Nanoparticle formulation results

Nanoencapsulation is a technology for creating particles at a small scale by coating substances through various methods. The coated particles, which become nanoscale in solid or liquid phases, are also called nanocapsules or nanoparticles [30, 31]. Nanoparticles are the result or product of nanoencapsulation techniques with a size of<1 µm [32]. To address the water solubility limitations of some organic solutes, functional organic compounds are often encapsulated within nanoparticles [33]. In this study, the method used for nanoparticle preparation was the ionic gelation method. The chitosan solution, extract, and Na-TPP were mixed homogenously to produce nanoparticles with a nanometer size. The chitosan solution concentrations varied at 0.1%, 0.2%, 0.3%, and 0.4%, while red bulb leaves ethanol extract and Na-TPP were kept constant at 8 mg/ml and 0.1%, respectively. The chitosan solutions were prepared by

dissolving 100 mg, 200 mg, 300 mg, and 400 mg of chitosan into 100 ml of 1% acetic acid. The 1% acetic acid solution was made by adding 1 ml of acetic acid to a 100 ml volumetric flask containing some distilled water, then filling it with distilled water to the mark and mixing gently. The 0.1% Na-TPP solution was prepared by dissolving 100 mg of Na-TPP in 100 ml of distilled water.

Nanoparticle characterization

Subsequently, the nanoparticles were characterized as a suspension at 1500 rpm for 5 h at room temperature. Stirring for 5 h was carried out because the formula showed different results with that stirring time. Visual observation revealed no floating particles or sediment in formulas 1, 2, and 3. The characterization aimed to determine which formulation met the criteria for suitable nanoparticles. The characterization included particle size measurements, polydispersity index, zeta potential, particle morphology, and the percentage inhibition of the free nanoparticle extract.

The desired particle size for nanoparticles is within the range of 100 to 300 nm. Particles within this size range are more easily able to penetrate cell membranes, making them suitable for drug-delivery systems [34-37]

The polydispersity index value indicates a homogeneous (monodisperse) particle distribution between 0 and 0.5 [38]. A monodisperse system is chosen because if the particle size distributed homogeneously, it will be easier to predict the drug absorption profile and facilitate the drug-delivery system.

The formula selection system uses an elimination method to exclude formulas that do not fall within the desired range for each criterion. Formulas that fall within the desired particle size range (100–300 nm) and have a polydispersity index in the range of 0–0.5 will undergo further characterization, explicitly determining the zeta potential of these formulas. Zeta potential measurements are performed to determine the charge contained in the nanosuspension. After measuring the zeta potential, the formula will be further prepared to assess the % inhibition of the free extract against DPPH. The formula with the lowest % inhibition of the free extract against DPPH will examine its partcile morphology using SEM, which will help identify the optimal chitosan concentration for formulating red bulb leaves ethanol extract nanoparticles.

Table 2: Characterization results of chitosan-nano encapsulated red bulb leaves ethanol extract

Formula	Particle size (nm)	Polydispersity index	Zeta potential (mV)	% Entrapment efficiency*	Inhibition test of free nanoparticle extract against DPPH (%)*
F1	265.1±67.1	0.177	1.10	67.592±1.15	22.328±0.794
F2	271.7±69.1	0.208	0.43	74.088±1.52	17.853±1.048
F3	299.8±79.8	0.194	0.31	77.114±1.13	15.768±0.780

*Results are expressed as a mean±SD, n=3

Particle size is a crucial initial parameter in nanoparticles. Generally, nanoparticle formulations must fall within the nano size range<1000 nm. The purpose of measuring particle size is to determine the size of the particles in the formulated product and to select which formulations are suitable for further testing. This study's target particle size is 100-300 nm, meaning that the particle size measurements of each formulation must fall within this range; otherwise, the formulation considered outside the desired nanosuspension criteria and will be eliminated. The results from particle size analysis (PSA) are shown in table 6. The particle sizes for F1, F2, and F3 are 265.1 nm, 271.7 nm, 299.8 nm, and 506.8 nm, respectively. F1, F2, and F3 fall within the desired particle size range and can be used for further testing, specifically zeta potential analysis.

The measurement results show that increasing the chitosan concentration affects particle size. The higher the concentration of chitosan used in the formula, the larger the particle size [39]. Fig. 1 shows the relationship between the addition of chitosan concentration and the increase in particle size. The more chitosan added, the greater the opportunity for the active substance to interact with the free polymer, resulting in larger particle sizes.

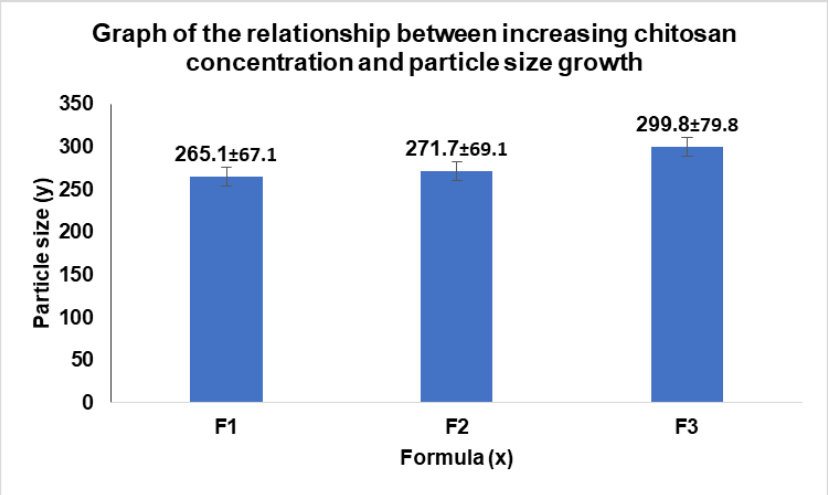

Fig. 1: Graph of the relationship between increasing chitosan concentration and particle size growth (Results are expressed as a mean±SD, n=3), Description: F1 = Chitosan 0,1%; F2 = Chitosan 0,2% and F3 = Chitosan 0,3%

In this study, the F1, F2, and F3 particles exhibited good size uniformity/homogeneity, with polydispersity index values of 0.177, 0.208, and 0.194, respectively.

Zeta potential analysis is a technique used to determine the charge on the surface of nanoparticles in a solution. Determining the zeta potential value assesses the particle charge and the stability of the

particles in the solution [40]. The zeta potential values obtained in this study for F1, F2, and F3 were 1.10, 0.43, and 0.31 mV, respectively.

Several factors, including the residual amino groups from chitosan, the charge of the active substance, and the pH of the formulation, can influence the zeta potential value. A high (positive) zeta potential value may be caused by residual free amino groups that have not yet interacted with the active substance or the cross-linker [38]. This study's small zeta potential values (close to zero) indicate very few free amine groups in the resulting nanoparticle suspension. This suggests that almost all amine groups have interacted with the active substance and the cross-linker.

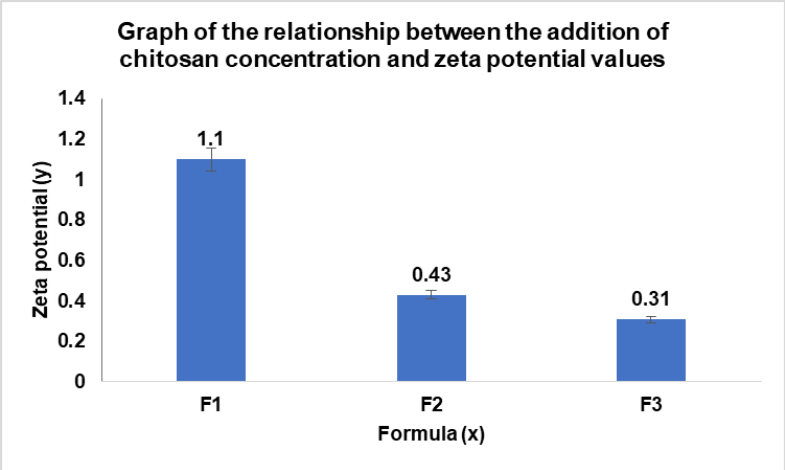

Fig. 2: Graph of the relationship between the addition of chitosan concentration and zeta potential values (Results are expressed as a mean±SD, n=3), Description: F1 = Chitosan 0,1%; F2 = Chitosan 0,2% and F3 = Chitosan 0,3%

A suspension pH outside the range of 4–7.5 will result in high zeta potential (>+30/-30 mV), making the particles more stable because the repulsive forces are more significant than the attractive forces. Strong repulsive forces prevent the particles from quickly forming aggregates, leading to more excellent stability. The zeta potential of the suspension will be low if the pH is within the range of 4–7.5. Especially if the pH of the formulation is at the isoelectric point, which is 5.5, the zeta potential value will approach zero [41]. This is consistent with the research results, where the pH values for F1, F2, and F3 fall within the range of 4 to 7.5, causing the zeta potential values to approach zero. Based on the zeta potential values, the resulting suspension can be considered unstable due to the low zeta potential. However, after observation over 7 d, no sedimentation was observed in F1, F2, or F3, indicating that the formulations tend to remain stable during 7 d of storage. The red bulb nanoparticle is stored in a temperature-controlled environment between 2–8 °C and is protected from light.

The entrapment efficiency of the extracts was assessed indirectly by measuring the quantity of extract that was not encapsulated in the nanoparticles. The total amount of the drug in each formulation was determined spectrophotometrically at 515.5 nm [42, 43]. Naphtoquinones were chosen as the reference standard for their relevance to the flavonoid content found in red bulbs. In this study, the entrapment efficiency in F1, F2, and F3, respectively, are 67.592±1.15%, 74.088±1.52% and 77.114±1.13%. The high percentage of entrapment efficiency means the higher the bioavailability of the active substance. This means that F3 has the highest bioavailability among the formulations due to its superior entrapment efficiency. This suggests that a greater proportion of the active substance is encapsulated within the nanoparticles in F3, enhancing its potential therapeutic effectiveness compared to F1 and F2. The nanoparticle morphology was observed on F3 because F3 had the lowest percentage inhibition of the free extract. The nanoparticle morphology was observed using SEM (Scanning Electron Microscopy). The F3 nanoparticle suspension samples were first dried using a freeze-drying method. Solvent removal was necessary because if there is still water or solvent in the sample, it will affect the imaging by showing water on the outer surface of the measured particles, making it difficult to determine the true particle morphology.

The imaging results obtained with SEM show that many particles cluster together (not separated). This may be influenced by the previous freeze-drying process, which caused the particles to aggregate. SEM is less effective in depicting the actual condition of the particles when in suspension.

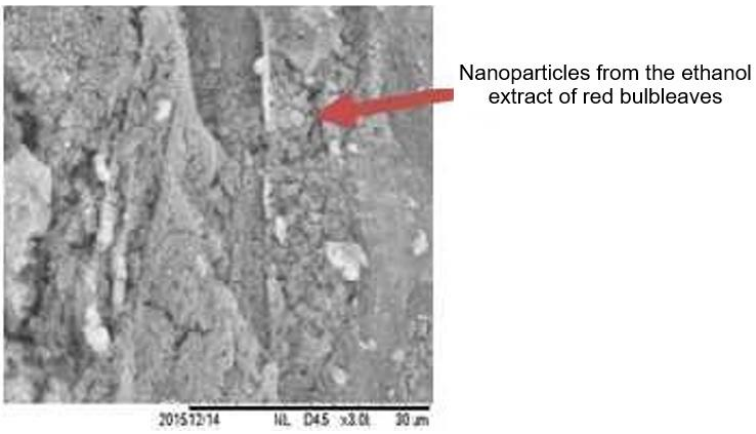

Fig. 3: Results of nanoparticle morphology testing with SEM

Verification of the spectrophotometric method with DPPH

Verification of a method involves confirming its validity by testing and providing objective evidence [44]. Method verification aims to ensure that the process meets the established requirements. In this study, method verification was performed on DPPH at various concentrations, with absorbance measurements taken using a visible spectrophotometer.

The maximum wavelength for DPPH was determined using a 30 ppm DPPH standard solution. Scanning for the maximum wavelength was performed within the 500-550 nm range with a measurement interval of 0.1 nm. The scanning results showed that the maximum wavelength for DPPH used was 515.5 nm. The wavelength for DPPH is 517 nm [21]. The results of the maximum wavelength measurements are acceptable if the obtained wavelength does not differ by more than ±2 nm from the pre-determined wavelength in the absorption region above 320 nm. Therefore, it can be stated that the obtained maximum wavelength measurements are acceptable [19].

Linearity is an analytical parameter that describes an instrument's ability to obtain test results that are proportional to the concentration of the analyte in the sample over a specific range of concentrations. The calculated correlation coefficient (r) for the average absorbance of each concentration in 3 replicates is 0.9998, with the linear equation Y=0.0210x–0.0564. The measurements are linear because the correlation coefficient is close to ±1.

Results of the percent inhibition test of free nanoparticle extract against DPPH

The percentage inhibition of free extract nanoparticles against DPPH is a method used to determine the amount of free extract within nanoparticles capable of inhibiting DPPH. The purpose of measuring the percentage inhibition of free extract in nanoparticles in this study is to provide an overview of the amount of extract that fails to interact with the chitosan polymer. The method used to measure the percentage inhibition of free extract nanoparticles in this study involves measuring the absorbance of DPPH remaining after interacting with the free extract within the nanoparticles.

The nanoparticle suspension is prepared first before measuring the percentage inhibition of the free nanoparticle extract. The nanoparticle suspension is placed into a microtube and centrifuged at 8000 rpm for 15 min [22]. This is done to separate the red bulb leaves extract that is free/not adsorbed by the polymer. The principle of separation during centrifugation is based on molecular weight differences. When centrifuged, the free extract that does not interact with the polymer will be in the filtrate (upper part) because it has a smaller molecular weight than the extract that successfully interacts with the polymer. The interaction between the extract and the polymer increases the molecular weight, so during centrifugation, the adsorbed extract will be at the bottom. After centrifugation, the solvent in the obtained filtrate is removed using freeze-drying. Freeze-drying is relatively safe and does not damage active compounds because it does not involve high-temperature heating to remove the solvent. The purpose of freeze-drying the obtained filtrate is to remove any remaining solvent, which could interfere with the interaction between DPPH and the free extract.

The free extract was then dissolved in methanol and mixed with 2 ml of DPPH. Subsequently, an incubation was carried out to maximize the interaction between the extract and DPPH. The incubation lasted 30 min in a dark environment and container. The treated samples were then measured for absorbance using a visible spectrophotometer with a wavelength of 515.5 nm. The % inhibition values of the free extract against DPPH from the study results were 22.328±0.794% for Formula 1, 17.853±1.048% for Formula 2, and 15.768±0.780% for Formula 3. The formula with the lowest % inhibition was formula 3, with a chitosan concentration of 0.3%. The % inhibition of the free extract in nanoparticles provides an overview of the amount of active compounds successfully encapsulated by the polymer. A lower % inhibition by the free extract indicates fewer active compounds are accessible in the formula, suggesting that the polymer encapsulates more extract.

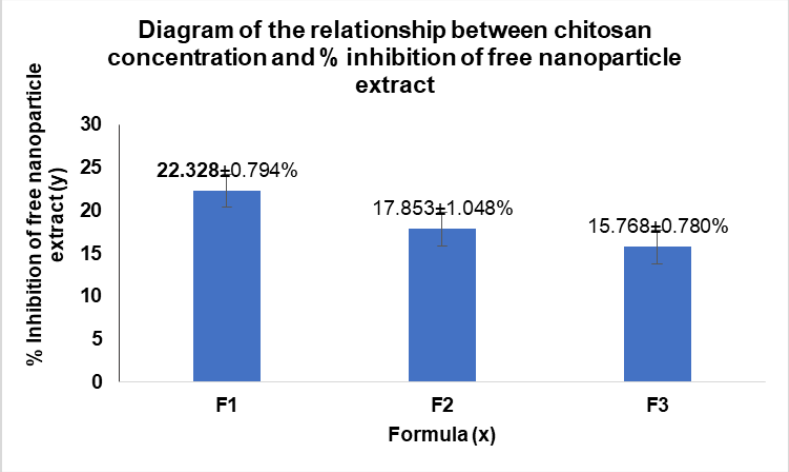

Fig. 4: Diagram of the relationship between chitosan concentration and % inhibition of free nanoparticle extract, (Results are expressed as a mean±SD, n=3), Description: F1 = Chitosan 0,1%; F2 = Chitosan 0,2% and F3 = Chitosan 0,3%

CONCLUSION

Based on the research conducted, the formulation of nanoparticles from the ethanol extract of red bulb leaves using chitosan at concentrations of 0.1-0.4% can be used to formulate of chitosan-ethanol extract nanoparticles. The formulation that produced nanoparticles with the best characterization was F3, with a particle size of 299.8 nm, a polydispersity index of 0.194, a zeta potential of 0.31 mV, and less spherical particle morphology.

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AUTHORS CONTRIBUTIONS

Wintari Taurina contributed in conceptualized and designed the study, gathered and analyzed the data, and drafted the initial manuscript. Involved in the interpretation of the data and provided critical revisions to the content. Mohamad Andrie assisted in data collection and contributed to data analysis, provided substantial input in the interpretation of the results, and reviewed and revised the manuscript for intellectual content. Each author has read and approved the final version of the manuscript.

CONFLICT OF INTERESTS

The author declares no conflict of interest

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