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THE DEVELOPMENT OF CHITOSAN NANOPARTICLES FROM *HIBISCUS SABDARIFFA* L CALYX EXTRACT FROM INDONESIA AND THAILAND

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ABSTRACT: Rosella (*Hibiscus sabdariffa* L) calyx has been reported to have an antioxidant activity. Anthocyanidin is the major compound of rosella and has low bioavailability as an ionic form. Rosella extract is an acceptable due to high acidity. Development of chitosan nanoparticle from *Hibiscus sabdariffa* extract were expected to improve the bioavailability and the acidity problem. We studied the optimum pH and composition ratios of chitosan, extract and tripolyphosphate (TPP) to develop chitosan-nanoparticle of rosella extract. We used rosella calyx both from Indonesia and Thailand to compare the physical characteristic the extract and nanoparticles. Rosella was extracted using 60% ethanol and water, followed by evaporation using evaporator and dried by freeze-dryer. The optimum formation of chitosan-nanoparticles of rosella extract were observed including pH formation and composition ratios between extract: chitosan: TPP. The physical characteristics of nanoparticles were observed using transmission electron microscope (TEM) and particle size analyzer (PSA). We found that optimum composition of extract: chitosan : TPP ratios were 2 : 1 : 0.1, with optimum formation of chitosan in acetate buffer pH 4. The characteristic of nanoparticles were spherical shapes with particles size range 60 to 300 nm, polydispersity index around 0.3 and zeta potential 39.19 to 62.26 mV. The nanoparticles has high entrapment efficiency of flavonoid active compound of 81.98 to 83.83%.

INTRODUCTION: The calyx of *Hibiscus sabdariffa* (roselle) has been used traditionally for curing various diseases¹. Rosella calyx extract have been reported as antioxidant², immunomodulator³, antidepressant⁴, antihyperlipidemia⁵, and⁶ diabetic⁶, and hepatoprotective agent⁷. Various antioxidant constituents have been found in the calyx of *Hibiscus sabdariffa* including anthocyanin, β -carotene, β -sitosterol, hibiscetin, hibiscin, hibiscitrin, and quercetin¹.

Rosella calyx extract has a high acidity level. This causes the anthocyanin are in the ionic form and lower bioavailability. High acidity level also contributes to the disruption of gastrointestinal mucosal in daily use, and making it unacceptable. Nanoparticles are defined as particulate dispersions (colloidal system) or solid particles with a size in the range of 10-1000nm⁸. Nanoparticles formulation is needed to increase the bioavailability of active compounds penetration. It also improve properties of poor solubility drug, instability, poor bioavailability, and potentially strong side effects that require drug enrichment at the site of action⁹.

The ionic gelation is the widely method was used to develop nanoparticle formulation¹⁰, as the simple preparation and the final nanoparticles has a

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positive charge. Chitosan is used together with tripolyphosphate (TPP) in various nanoparticle preparation by ionic gelation method¹⁰. The previous research reported that nanoparticles prepared by chitosan as a polymer and TPP found to discrete particles of nanosize range with good stability^{11,12}.

The objective of this study were to obtain the optimal formula based on composition of chitosan, rosella calyx extract, the concentration of tripolyphosphate (TPP) and the pH degrees of acetate buffer on dissolution of chitosan in preparation of nanoparticles and followed by characterization.

MATERIAL AND METHOD:

Collection of material:

The rosella calyx were collected from Madiun (East Java, Indonesia) and Narathiwat (Southern Thailand). After it had been dried were pulverized and stored in a desiccator before extraction.

Extraction:

The extraction of dried rosella calyx were performed using maceration method with 60% ethanol and boiling method with water for one hour. After this process the ethanol extract and water extract was found. Each extract was evaporated to get the concentrated extract and followed by freeze dryer.

Optimization of extract and chitosan ratio:

Chitosan nanoparticles were prepared based on ionic gelation method. Optimization of composition between chitosan and rosella extract was carried out by mixing chitosan solution and rosella extract in ratio: 1/3, 1/2, 1/1, 2/1, or 3/1 (mass ratios) in phosphate buffer solution pH 4.0. The mixture was homogenized using vortex for 30 seconds and then add TPP solution in a final concentration of 0.2 mg/ml. The dispersion of nanoparticles was observed for 7 days. The optimum formula were found if there was no precipitation (Brown Motion).

Optimization of tripolyphosphate ratio:

After the optimization ratio of chitosan and rosella extract has been carried out, then was followed by optimization of TPP ratio. Various concentrations

of TPP (0.2, 1, 2 mg/ml) were prepared in aqueous solution. The optimum of TPP ratio was observed in 1/10, 1/2, and 1/1 mass ratios between chitosan (with extract) and TPP. The best ratio was observed by the absence of precipitation. The observation was carried out during 7 days.

Optimization of phosphate buffer pH:

After the optimum ratio between rosella extract:chitosan:TPP was found then was followed by studying on the buffer pH. The various buffer pH use were 4.0, 5.0, and 6.0. The optimum pH was observed in the absence of precipitation during 7 days.

Nanoparticle characterization:

The optimum nanoparticle formula was characterized by the morphology and particles size using transmission electron microscope (TEM) (JOEL 5C-560, Japan) and particle size analyzer (PSA) DelsaTM Nano Submicron Particle Size Analyser (Beckman Coulter).

Determining Entrapment Efficiency:

The entrapment efficiency of *H.sabdariffa* active compound was carried out by determination of total flavonoid concentration. The nanoparticles were centrifuged at 10,000 RPM for 30 min. The concentration of total flavonoid in the supernatant was quantified using quercetin standard. The low concentration found in the supernatant showed the high entrapment efficiency in the chitosan nanoparticles. The entrapment efficiency (EE) was calculated using this formula:

$$\% EE = \frac{\text{Flavonoid Total} - \text{Free Flavonoid (Supernatant)}}{\text{Flavonoid Total}} \times 100 \%$$

RESULT AND DISCUSSION:

The optimum formula of chitosan nanoparticles of *H.sabdariffa* extract:

Chitosan nanoparticles were prepared based on ionic gelation method. The *H. sabdariffa* extract including water, and ethanolic extract from Indonesia and Thailand were observed. The previous study reported that characterization of chitosan nanoparticles was significantly influenced by the ratio between extrat, chitosan and TPP^{13,14}.

The study found that rosella extract:chitosan:TPP ratio was 2:1:0.1. The ratio of chitosan needed was less than the extract due to low concentration, the gelation medium has low viscosity. The low viscosity could supply the better medium for nanoparticle formation¹⁵. The smaller nanoparticles may be attributed to a greater cross-linking density by the interaction between chitosan matrix and the flavonoid groups in rosella calyx. The formula ratio of TPP was 0.1 less than extract and chitosan. The large amount of TPP gives the precipitation of nanoparticles due to the bigger size particle formation (microparticle size)¹⁶.

pH Optimization:

Acetate buffer was used to dissolve chitosan. This study was found that the pH 4 was the optimum pH. Previous research found that size of nanoparticles decreased with increasing of pH, indicating an influence of pH on particle size¹⁴. In this study we found that association between extract, chitosan and TPP is driven by pH. The charge degree of both chitosan and TPP were influenced by pH value¹¹. At a critical low pH, most of the amino groups of chitosan are protonated, enabling the chitosan molecule with an

extension confirmation due to strong charge repulsion.

The TPP molecule is also protonated, leading to lower charging density of the molecule. In this case, the chitosan molecules cannot be sufficiently cross-linked by TPP to form stable particles. Along with increase of the pH value, the deprotonation degree of TPP is increased gradually, while the protonation degree of chitosan is less influenced when the pH value is below 5. At a proper pH such as pH 3.5, the charge interaction between these two molecules becomes strong enough, thus stable chitosan nanoparticles were obtained with a relatively larger size.

At a higher pH value, the charging degree of TPP molecule is enhanced again, which neutralizes the chitosan to a greater extent. Consequently, the particles shrink their size again. Protonated amino group on acid solvent could bind effectively to the active ingredient, thus forming stable nanoparticles and having a small particle size¹⁴.

The hypothetic interaction between chitosan, TPP and cyanidin structure were shown in Figure 1. The interaction involved the electrostatic and hydrogen bonding interaction.

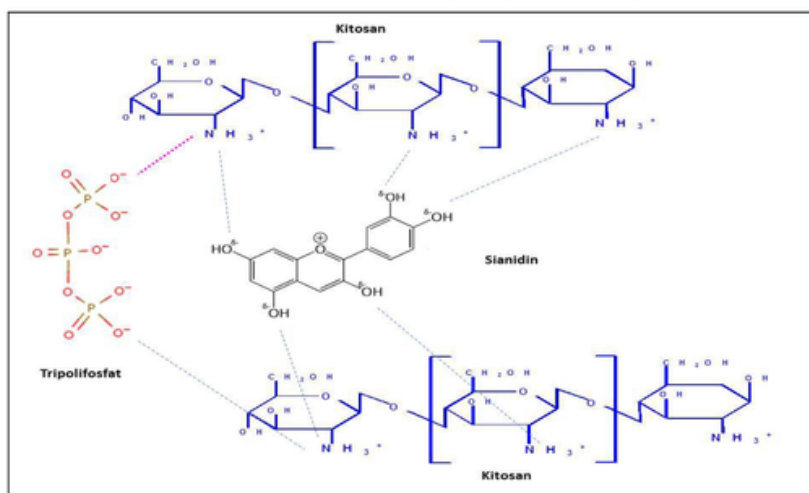


FIG. 1: HYPOTHETIC INTERACTION BETWEEN CHITOSAN, TPP AND CYANIDIN

The interactions of chitosan nanoparticle formulations and rosella extract is the electrostatic interaction between the positively charged amine groups of chitosan which protonated under acidic conditions and oxygen atoms in the structure of the flavonoid/polyphenol compounds which are The TPP stabilized this interaction.

Characterization of nanoparticles:

Characterization of nanoparticles using TEM showed a spherical shape (Figure 2). The particle size distribution of nanoparticles was shown by Figure 3.

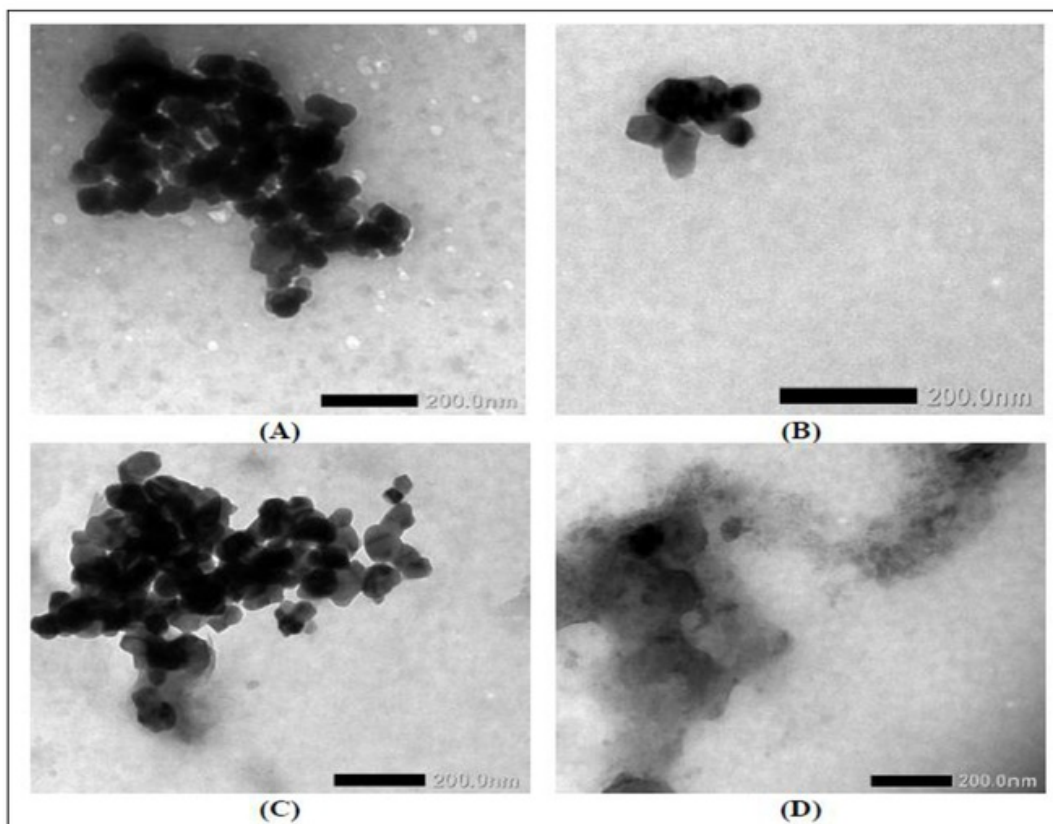


FIG.2: THE SHAPE OF NANOPARTICLES FROM *H SABDARIFFA L* EXTRACT. A. THAI ETANOLIC EXTRACT, B. INDONESIA ETHANOLIC EXTRACT, C., THAI WATER EXTRACT, D. INDONESIA WATER EXTRACT.

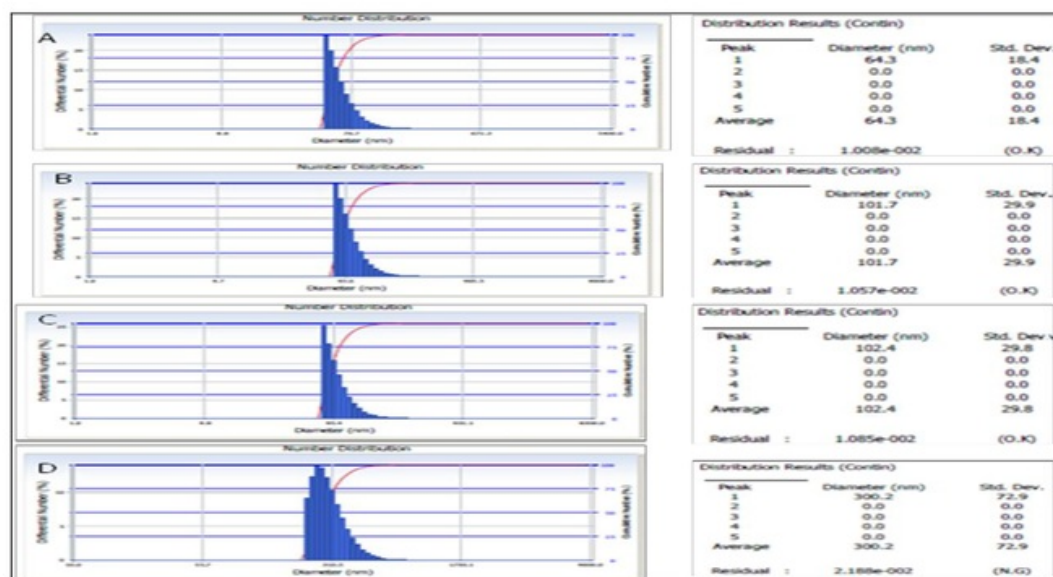


FIG. 3: THE PARTICLE SIZE DISTRIBUTION OF NANOPARTICLE OBSERVED USING PARTICLE SIZE ANALYSER. A. THAI ETANOLIC EXTRACT, B. INDONESIA ETHANOLIC EXTRACT, C., THAI WATER EXTRACT, D. INDONESIA WATER EXTRACT.

The nanoparticle size found in this research were in the range of nanoparticles (60-300 nm). Previous

study on development of nanoparticle from *Garcinia mangostana* extract found the particle

size distribution were 200-500nm¹⁶. Formulation of curcumin nanoparticle found the particle size of 269.8 nm¹³. Small particle size increase surface area, thereby increasing the absorption and stability of the nanoparticles¹².

The characteristic of nanoparticles could be analyzed with polydispersity index. Polydispersity index describes the distribution of particle size present in the preparation of nanoparticles, the smaller the number of polydispersity index, the more uniform the size of the particles. If there is a significant difference of size between the larger particles and the smaller ones, it will affect the particles' characteristic. The larger the size of the particles, the particle will precipitate more easily. The polydispersity index value less than 0.5 were accepted¹⁵. The particle size and the polydispersity index were shown in Table 1. This data indicates that nanoparticles in this study have a uniform size.

The stability of nanoparticles were shown by zeta potential value. Zeta potential is used to evaluate the stability of nanoparticles. Zeta potential is a parameter of the surface charge of particles that affect the stability. Zeta potential values showed levels of particles to repel to each other. It depends on the chemical properties of the polymer (chitosan), stabilizing agents (tripolyphosphate), the pH of the medium, and the properties of the active ingredient¹⁷. Bigger zeta potential showed the stable suspension.

The zeta value of +/-30 showed the stable suspension as the charge on the surface of the particles prevents aggregation. The zeta potential value were shown in Table 1 and Figure 4. Zeta potential values of the four extract formulations of nanoparticles found in this study has a good physical characteristic.

TABLE 1: THE AVERAGE OF PARTICLE SIZE AND POLYDISPERSION INDEX OF CHITOSAN NANOPARTICLE *H.SABDARIFFA* L EXTRACT

S. No	Extract	Average particle size	Polidispersion index	Zeta potential
1	Thai Ethanolic Extract	64.3 ± 18.4	0.316	54,27
2	Ind Ethanolic Extract	101.7 ± 29.9	0.309	62,26
3	Thai water Extract	102.4 ± 29.8	0.315	-53,49;
4	Ind water Extract	300.2 ± 79.2	0.263	-39,19

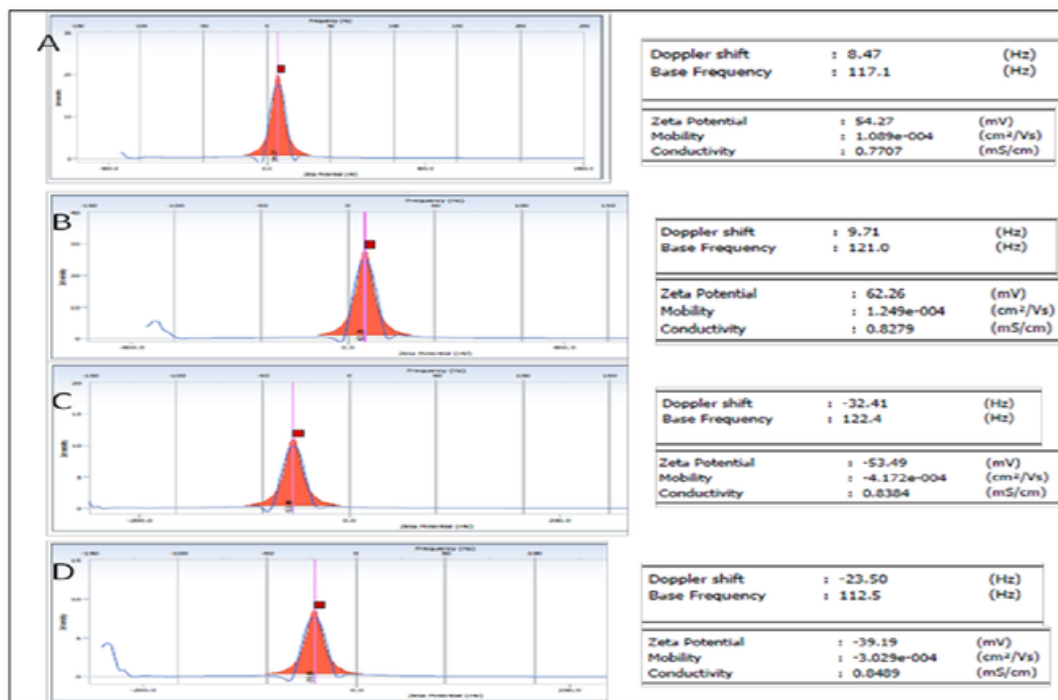


FIG. 4: ZETA POTENTIAL VALUE OF CHITOSAN NANOPARTICLES A. THAI ETANOLIC EXTRACT, B. INDONESIA ETHANOLIC EXTRACT, C., THAI WATER EXTRACT, D. INDONESIA WATER EXTRACT.

Entrapment efficiency:

Nanoparticle system has the capability of high Entrapment Efficiency. Entrapment Efficiency was influenced by drug interactions with polymers and ratio of polymer compositions¹⁸. The entrapment efficiency was determined by the different concentration of free polyphenols and entrapped in the nanoparticles after precipitation with centrifugation. In this study, the Entrapment Efficiency was found more than 80% (Table 2). Previous study reported the Entrapment efficiency of rose hips nanoparticle of 25.8%-46%¹⁹. And Entrapment Efficiency values of nanocurcumin about more than 70%¹³.

The entrapment Efficiency was affected by the solubility of the active ingredient in a polymer matrix, polymer composition used, molecular weight, interaction of active ingredient with the polymer, and the presence of functional groups⁸.

TABLE 2: THE ENTRAPMENT EFFICIENCY OF CHITOSAN NANOPARTICLES H SABDARIFFA EXTRACT

No	Extract	Entrapment Efficiency
1	Thai Ethanolic Extr	82,21 %
2	Ind Ethanolic Extr	83,83%
3	Thai water Extr	81,98%
4	Ind water Extr	82,18%

CONCLUSION: The results showed that rosella extracts: chitosan: tripolyphosphate optimum ratio was 2: 1: 0.1, with optimum dissolution of chitosan in acetate buffer pH 4 and at this pH, the chitosan was the optimum to dissolve as well. The nanoparticles has a good characteristic including the spherical shape, the average particle size of chitosan nanoparticles ethanol extract at 60-300 nm, zeta potential and high Entrapment Efficiency.

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