

Development of Nanoparticles Pegagan Leaves Ethanolic Extract (*Centella asiatica* (L.) Urban) Using Variation Concentration of Poly-Lactic-Co-Glycolic Acid (PLGA) Polymer

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ABSTRACT

Pegagan is a plant that plays an important role in health because of its secondary metabolite. However, many secondary metabolites tend to be unstable when exposed to UV light and oxygen such as flavonoid and terpenoid. The purpose of this study was to formulate the ethanolic extract of pegagan leaves into nanoparticle preparations to increase the stability of the extract. Nanoparticle preparations were made using the emulsion solvent evaporation method using Poly-Lactic-Co-Glycolic Acid (PLGA) and polyvinyl alcohol (PVA). PLGA acts as a polymer that will coat the extract and PVA as a stabilizer. Variations in the concentration of PLGA used were 50 mg, 75 mg, and 100 mg, while the concentration of PVA used was 40 mg and the extract concentration was 158 mg. Determination of the best formula is done by looking at the results of the percent encapsulation efficiency obtained from the three formulas, namely 93.68%, 85.35%, and 88.76%, respectively. Based on these results, formula 1 was determined as the best formula. The particle size obtained in the best formula was 288.1667 ± 3.4195 nm, the polydispersity index (PDI) was 0.371 ± 0.0045 and the zeta potential value was -10.6333 ± 0.1154 . A physical stability test (cycling test method) of the best formula found a decrease in pH of 0.54 and no organoleptic changes or precipitate formed.

Keywords: Pegagan leaves ethanolic extract; PLGA; PVA; Nanoparticles

INTRODUCTION

Pegagan is a plant from the Apiaceae family that grows in tropical areas such as Indonesia. Pegagan is known to have many pharmacological activities. The most widely used part of the plant is the leaves. Pegagan leaves are known to have activity as an anti-inflammatory (Ratz-Łyko *et al.*, 2016), neuroprotective (Khan and Orhan, 2012), wound healing (Irhan *et al.*, 2019), antioxidant (Park *et al.*, 2017), urinary tract disease. respiratory disease (Chandrika and Kumara 2015), skin diseases (Bylka *et al.*, 2014), antibacterial (Nasution *et al.*, 2018), etc. The pharmacological activity of pegagan leaves is related to their secondary metabolites. Pegagan leaves contain flavonoid compounds, saponins, triterpenoids, alkaloids, phenolics, and glycosides (Gray *et al.*, 2018; Irham *et al.*, 2019). Pegagan leaves contain the most triterpenoid compounds such as asiaticoside, madecossida, asiatic acid, and centellosida (Irham *et al.*, 2019). In addition, pegagan leaves also contain lots of flavonoid compounds such as rutin, quercetin, kaempferol, and luteolin (Azmin and Nor, 2020). However, these secondary metabolites are easily unstable due to various factors such as oxygen and light.

Research by Ramesova *et al.* (2011) proved that quercetin and luteolin compounds are unstable when exposed to atmospheric oxygen which causes degradation. In addition, research conducted by Puttarak and Brantner (2016) proved that the triterpenoid asiaticoside content in pegagan leaves in the form of glycosides is unstable when exposed to light or oxygen, but the aglycone form is much more stable than the glycosides. The instability of these secondary metabolites will greatly affect their pharmacological activity so development is needed to increase the stability of the extract, one of which is encapsulated using polymers into nanoparticles.

Nanoparticles are a development of pharmaceutical technology that can produce small particles of 10-1000 nm. Nanoparticles can be made using polymers. In this study, the polymer used was Poly-Lactic-Co-Glycolic Acid (PLGA). PLGA will coat and protect the extract from environmental influences such as light and oxygen. The mechanism of PLGA in coating the extract is by binding the extract with the functional group of PLGA so that nanospheres nanoparticles are formed. Each of these nanospheres contains active extract substances that have been coated by PLGA so that the extract has good stability (Prasetyo, 2017). In the manufacture of these nanoparticles, Polyvinyl alcohol (PVA) is also used as a stabilizer.

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PVA will act as an emulsifier between the oil phase and the water phase in the nanoparticle system made using the Emulsion Solvent Evaporation Method. The combination of PLGA-PVA has a strong sigma bond so that it will protect the encapsulated compounds in it (Kemala *et al.*, 2012). In addition to increasing the stability of the extract, the particle size produced in the manufacture of nanoparticles will also increase its pharmacological effectiveness. The size of the nanoparticles will make it easier for compounds to penetrate the intercellular space so that they can increase their penetration to target cells (Buzea *et al.*, 2007).

Based on the description above, the researchers are interested to formulating the ethanolic extract of pegagan leaves into nanoparticles using PLGA as a polymer and PVA as a stabilizer with the Emulsion Solvent Evaporation Method. This study will produce 3 formulas with variations in the concentration of PLGA, namely 50 mg, 75 mg, and 100 mg (Mardiyanto *et al.*, 2018). Determination of the best formula is based on the percentage of encapsulation efficiency (%EE) obtained. The best formula was then characterized in the form of particle size, polydispersity index (PDI), and zeta potential. The best formula will also be tested for stability using the Cycling Test method.

METHODOLOGY

Materials

The materials used in this study include Pegagan leaves (*Centela asiatica* (L.) Urban) (Batang, Central Java, Indonesia), PLGA (Sigma Aldrich®, Singapore), PVA (Sigma Aldrich®, Singapore), ethanol 96% (PT Dira Sonita®, South Sumatra, Indonesia), aquadest (PT Dira Sonita®, South Sumatra, Indonesia), ethyl acetate (Merck®, Jakarta, Indonesia), quercetin (Sigma Aldrich®, Singapore), methanol pa (Merck®, Jakarta, Indonesia).

Tools

The research was carried out using tools including scales, analytical (Ohaus®) 0.0001 g, rotary evaporator (Yamato®RE301), spectrophotometer UV-Vis (Biobase® BK-UV1900PC), glassware (Pyrex®), centrifugation, pipette micro (Dragonlab®), sonicator, spin bar (Scienceware®), magnetic stirrer (IKA® C-MAG HS 4) and Particle Size Analyzer (Malvern).

Methods

Preparation of Ethanolic Extract

Pegagan leaves are obtained from Central Java, Indonesia. Pegagan leaves are cleaned with

running water and then dried in the sun and covered with black cloth to obtain *simplicia*. Dried *simplicia* in a blender until a fine powder is obtained. The powder is then soaked into 96% ethanol in a ratio of 1:5 (Apriani *et al.*, 2021). Maceration was carried out for 5 days and stirred every 6 hours. This procedure was carried out 2 times. The resulting macerate is then concentrated using a rotary evaporator to obtain a thick extract.

Phytochemical Screening

Phytochemical screening was carried out to identify flavonoids, phenolic compounds, alkaloids, steroids, triterpenoids, saponins, and tannins. Flavonoid testing was carried out using concentrated HCl and Mg powder, positive results were indicated by the presence of a dark red color. Phenolic examination was carried out using FeCl₃, a positive result was indicated by a change in color to green, red, purple or black. Alkaloids were tested using Mayer reagent, Wagner reagent and Dragendorff reagent. Formed white precipitate on Mayer's reagent, brown precipitate on Wagner's reagent, and orange precipitate on Dragendorff's reagent in each test result showed positive results containing alkaloids. Testing for steroids and triterpenoids was carried out using anhydrous acetic acid and concentrated sulfuric acid, if green color is formed, it means that it is positive for steroids, while if it is red or orange, it is positive for triterpenoids. The saponin examination was carried out by vigorously shaking the extract solution, if the foam was formed, it was positive for saponins. The procedure carried out refers to the WHO guidelines and Harborne (WHO, 1998; Harborne, 1984)

Determination of Total Flavonoid Content

Total flavonoids content was measured using the aluminum trichloride colorimetric method (Indarti *et al.*, 2019). Quercetin was used as a standard for comparison to create a calibration curve. One milliliter of quercetin was dissolved in 96% ethanol and diluted to 10; 15; 20; 25; and 30 µg/ml. The diluted standard solution was mixed with 0.1 mL of aluminum trichloride, 0.1 mL of 1 M potassium acetate, and up to 5 mL aquadest. Furthermore, the absorbance was measured at a maximum wavelength of 425 nm using a UV-Vis spectrophotometer. Similarly, 100 µg/mL ethanolic extract were reacted as described above and measure the absorbance. This procedure was replicated three times.

Formulation of Nanoparticles PLGA-Extract

The formula for nanoparticles PLGA-ethanolic extract of pegagan leaves refers to the

Table I. Formula of Nanoparticles PLGA-Extract

Ingredient	Formula		
	F1	F2	F3
Extract (mg)	158	158	158
PLGA (mg)	50	75	100
PVA (mg)	40	40	40
Aquadest ad (ml)	25	25	25

research of Mardiyanto *et al.* (2018) and Rahayu *et al.* (2020). The nanoparticles were made using the Emulsion Solvent Evaporation Method. This study obtained 3 formulas with variations in PLGA concentrations of 50 mg, 75 mg, and 100 mg. The formula for PLGA Extract nanoparticles can be seen in Table I.

Nanoparticles PLGA-Extract were prepared by dissolving PLGA and extract in ethyl acetate and PVA in aquadest. PLGA solution and ethanolic extract were mixed as the oil phase. In a separate container, the PVA solution was prepared on a magnetic stirrer as the aqueous phase. The oil phase is dropped little by little into the water phase. This process was carried out for 1 hour with a homogenizer speed of 750 rpm. Then the solution was sonicated and aquadest was added up to 25 ml. The solution was then evaporated to remove the remaining organic solvent for 24 hours.

Determination of % Encapsulation Efficiency (EE)

Determination of the percent encapsulation efficiency was carried out using an indirect method referring to the research of Apriani *et al.* (2019). The suspension of nanoparticles was centrifuged at 9500 rpm for 1.5 hours. The supernatant was then measured for total flavonoid content according to the above procedure and the percent encapsulation efficiency was calculated using Equation 1.

$$\text{Encapsulation Efficiency (\%EE)} = \frac{T-S}{T} \times 100\% \dots\dots\dots [1]$$

Information: T: Total concentration of flavonoid in nanoparticle suspension ($\mu\text{g/mL}$); C: The concentration of flavonoid in the supernatant ($\mu\text{g/mL}$).

Characterization of Nanoparticle PLGA-Extract

The characterization of the nanoparticles carried out was the measurement of particle size, polydispersity index, and zeta potential using a Particle Size Analyzer (PSA) (Malvern, PT. DKSH

Indonesia) at 25°C with a scattering angle of 90° after diluted of 50 L suspension with 5 ml aquadest.

Stability Test

Stability test of nanoparticles was carried out using the cycling test method (Apriani *et al.*, 2018). The cycling stability test was carried out for 6 cycles where 1 cycle consisted of storing samples at a temperature of 4±2°C for 24 hours and then at a temperature of 40±2°C for 24 hours. Organoleptic observations (change in color, odor, and sediment) and pH were carried out in each cycle.

Data Analysis

Data analysis was performed using SPSS with one-way ANOVA method and paired t-test to see the difference in significance in each group.

RESULT AND DISCUSSION

Pegagan (*Centela asiatica* (L.) Urban) leaves were collected in Delisen, Sidalang, Kec. Tersono, Kab. Batang, Central Java. The dried simplicia of pegagan leaves were macerated using 96% ethanol as a solvent to collect a polar flavonoid compound. The yield of ethanolic extract of pegagan leaves was 9.16%. The ethanolic extract of pegagan leaves was screened for phytochemicals and the results showed that the ethanolic extract of pegagan leaves contained flavonoids, phenolics, alkaloids, terpenoids, saponins, and tannins as shown in Table II.

According to research conducted by Azmin and Nor (2020), the ethanolic extract of pegagan leaves was proven to contain flavonoid compounds such as kaempferol at 372.2 mg/g, quercetin at 77.6 mg/g, rutin at 15.0 mg/g, and luteolin at 2.7 mg/g. In this study, the flavonoid content obtained was 42.42 mg/g. Flavonoids are known to have various pharmacological effects such as antioxidants (Rana and Gulliya, 2019), anti-inflammatory (Maleki *et al.*, 2019), antibacterial (Xie *et al.*, 2015), anti-hypertension (Maaliki *et al.*, 2019), anti-diabetic (Al-Ishaq *et al.*, 2019), anticancer (Kopustinskiene *et al.*, 2020), etc. The pharmacological effect of pegagan leaves is

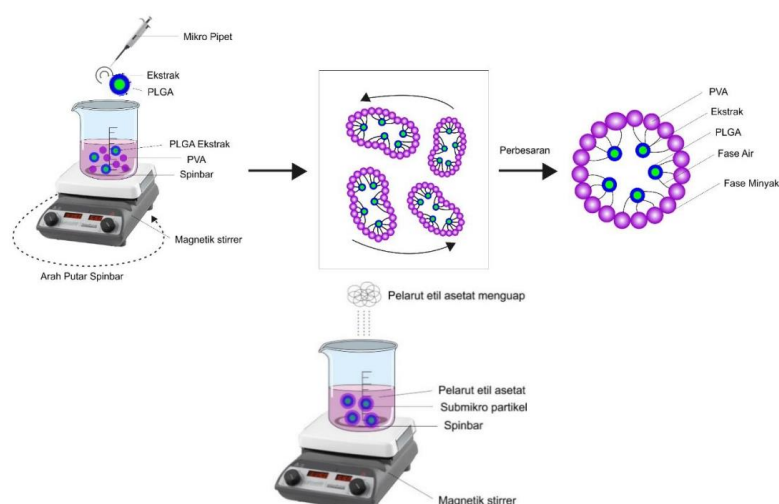


Figure 1. The Process of Nanoparticles Preparations

Table II. Phytochemical Screening Result

Secondary Metabolites	Result
Flavonoids	+
Phenolics	+
Alkaloids	+
Steroids	-
Terpenoids	+
Saponins	+
Tannins	+

strongly influenced by the stability of the secondary metabolites contained. Flavonoids are compounds that are easily degraded because they interact with the environment. Research by Ramesova *et al.* (2011) proved that quercetin and luteolin compounds are unstable when exposed to atmospheric oxygen which causes degradation. Research Sharma *et al.* (2015) and Ioannou *et al.* (2020), also proved that when flavonoids are exposed to high temperatures and light exposure, flavonoids will be degraded and reduce their effect.

In this study, the ethanolic extract of pegagan leaves was made in the form of nanoparticles using Poly-Lactic-Co-Glycolic Acid (PLGA) as polymers and Polyvinyl Alcohol (PVA) as stabilizers. PLGA used as the polymer because PLGA is biodegradable and biocompatible and the use of PLGA has been approved by the FDA. The nanoparticles were prepared using the Emulsion Solvent Evaporation Method. PLGA and extracts will act as the oil phase in the emulsion system while the aqueous phase is in the form of aquadest. PLGA will coat the extract because of the interaction between the carbonyl group and the carboxyl group on the flavonoid and polymer to form nanospheres (Pool *et al.*, 2012). Each of these

nanospheres contains active extracts that have been coated by PLGA. PVA acts as a surfactant because it contains a hydrophilic part that will bind to the water phase and a hydrophobic part that will bind to the oil phase (Jalalian *et al.*, 2016). The organic solvent used will then be evaporated to produce a suspension of PLGA-Extract nanoparticles as shown in Figure 1.

The nanoparticles were made into 3 formulas (F1, F2, and F3) with variations in PLGA concentration, namely 50 mg, 75 mg, and 100 mg. The resulting suspension of Nanoparticles PLGA-Extract is green where the more PLGA is used, the more concentrated the color of the suspension as shown in Figure 2.

The success of the encapsulation of the ethanolic extract of pegagan leaves on the PLGA polymer is reflected in the results of the % encapsulation efficiency. The results of the encapsulation efficiency can be seen in Table III. F1 has a higher encapsulation efficiency value than F2 and F3. The higher the concentration of PLGA used, the more surface area of the polymer that can encapsulate the extract (Ismail *et al.*, 2019). However, if the concentration of PLGA used is too much, it will make the solution more viscous so



Figure 2. Nanoparticles Suspension: F1 (50 mg PLGA), F2 (75 mg PLGA), dan F3 (100 mg PLGA)

Table III. The Result of % Encapsulation Efficiency

Formula	%EE \pm SD	%CV
F1	93.6809 \pm 0.2694	0.0029
F2	85.3473 \pm 0.1925	0.0023
F3	88.7603 \pm 0.1909	0.0022

Table IV. The Characterization Result of F1

Parameter	Average \pm SD
Particle Size (nm)	228.1667 \pm 3.4195
PDI	0.371 \pm 0.0123
Zeta Potential (mV)	-10.6333 \pm 0.0108

that the dispersion process of drug compounds will be difficult and result in lower drug absorption (Pakulska *et al.*, 2016). Based on the results of statistical analysis, there were significant differences in each treatment group ($p < 0.05$) so the difference in PLGA concentration significantly affected the resulting encapsulation efficiency.

Formula 1 was determined as the best formula based on the resulting encapsulation efficiency. F1 was then characterized in the form of particle size, polydispersity index, and zeta potential. The results of the characterization of F1 can be seen in Table IV. Based on table IV, the particle size of the nanoparticles PLGA-Extract obtained was 228.1667 \pm 3.4195 nm. Particle size determines the effectiveness of the active substance. The smaller the particle size, the permeability will increase so the penetration of the active substance into the target cell will be even greater (Hoshyar *et al.*, 2016; Mitchell *et al.*, 2021). The value of the polydispersity index also plays an important role in the penetration ability of nanoparticles. The polydispersity value describes the uniformity of particle size where if the PDI

value is more than 0.7 then the nanoparticles formed are said to be non-uniform (Danaei *et al.*, 2018). The PDI value obtained in this study was 0.371 \pm 0.0123, which means that the nanoparticles made have a uniform particle size. The nanoparticles PLGA-Extract also measured the zeta potential value to see the stability of the nanoparticles formed. A good zeta potential value for nanoparticles is greater than +30 mV or less than -30 mV, which means that the nanoparticles are strong cationic and strong anionic. The high zeta potential value indicates that the particle charge of the nanoparticles is high so that it will prevent the aggregation of due to electric repulsion (Samimi *et al.*, 2019). The zeta potential value in this study was -10.6333 \pm 0.0108 mV. Nanoparticles with a zeta potential value between -10 and +10 mV are included in the neutral category so that they can aggregation (Clogston & Patri, 2011). In addition, the zeta potential value can also describe the surface charge of the resulting nanoparticles. The surface charge will also describe the effectiveness and toxicity of nanoparticles against target cells (Rasmussen

Table V. The Stability Result of F1

Cycle	Organoleptic	pH \pm SD
0	Odorless, light green, no sediment	6.10 \pm 0.10
1	Odorless, light green, no sediment	5.93 \pm 0.05
2	Odorless, light green, no sediment	5.66 \pm 0.05
3	Odorless, light green, no sediment	5.60 \pm 0.10
4	Odorless, light green, no sediment	5.63 \pm 0.05
5	Odorless, light green, no sediment	5.50 \pm 0.10
6	Odorless, light green, no sediment	5.56 \pm 0.15

et al., 2020). Target cells usually have a negative charge so that nanoparticles that have cationic properties will show good effectiveness but also high toxicity (Clogston & Patri, 2011). In this study, nanoparticles with anionic charge were obtained because the PLGA polymer contained an anionic carboxyl group.

Formula 1 was also tested for stability using the heating-cooling cycle Method. Observations were made in each cycle and can be seen in Table V. Based on the results of statistical analysis with the paired t-test method, between the 0th and 6th cycles there was a significant difference ($p < 0.05$), which means that the decrease in pH occurred was quite significant. This decrease in pH was caused by the rupture of vesicles in PLGA and PVA which caused the release of flavonoids. The released flavonoids will undergo a degradation process (releasing H⁺ ions) which will cause the pH of the nanoparticles to become more acidic (Ramesova *et al.*, 2011). However, organoleptically there was no change from the 0th cycle to the 6th cycle.

CONCLUSION

The concentration of PLGA in the manufacture of nanoparticles had a significant effect on the percent value of encapsulation efficiency ($p < 0.05$). The smaller the PLGA concentration used, the greater the percentage of encapsulation efficiency obtained. F1 is the best formula where the percent encapsulation efficiency is 93.6809 ± 0.2694 and the particle size obtained is 228.1667 ± 3.4195 nm, the PDI value obtained is 0.371 ± 0.0045 , and the zeta potential value obtained is -10.6333 ± 0.1154 mV. The particle size and PDI values obtained in the best formula meet the requirements, while the zeta potential value tends to be less qualified because it is possible for aggregation to occur. The results of the stability test from F1 showed that there was a significant decrease in pH from cycle 0 to cycle 6 even though there was no change in organoleptic.

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