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In Vitro Anti-Hyaluronidase Activity Test of *Alpiniazerumbet* Rhizome Extract (*Alpiniazerumbet* (Pers.) B.L. Burtt& R.M. Sm) and Preparation of Solid Lipid Nanoparticle

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Abstract: ***Alpinia zerumbet rhizome (Alpinia zerumbet (Pers.) BL Burtt & RM Sm) contains phenolic compounds that have anti-hyaluronidase activity that can reduce wrinkles on the skin. This study aims to test anti-hyaluronidase activity of Alpinia zerumbet rhizome extract using in vitro method and to prepare it in the form of nanoparticles with Solid Lipid nanoparticles (SLN) delivery system. Powder of Alpinia zerumbet rhizome was extracted with 70% ethanol using kinetic maceration method, then concentrated by rotary vacuum evaporator at 100 mmHg at a temperature of ±50°C. Anti-hyaluronidase activity of extract was tested using in vitro method with ELISA Reader, and then prepared into nanoparticles with SLN delivery system. Extract loaded SLN were prepared using polyethyleneglycol 8-beeswax and acrylyl glucoside underhigh speed homogenization-ultrasonication methods. Afterwards, extract loaded SLN was characterized. The test results of anti-hyaluronidase activity (IC_{50}) of Alpinia zerumbet rhizome extract was 81.04 mg/mL. The characterization of Alpinia zerumbet rhizome extract loaded SLN were particle size, polydispersity index, zeta potential, and morphology analysis, all of the tests met the requirements of nanoparticle with results respectively, 138.6 nm; 0.226; -29.9 mV and a spherical shape. In conclusion, Alpinia zerumbet rhizome extract loaded SLN has potential as an anti-hyaluronidase agent with the ability to reduce wrinkles on the skin.***

Keywords: Alpiniazerumbet rhizome extract, Solid Lipid Nanoparticles, anti-hyaluronidase

1. Background

Skin is the main target of exposure to external agents by a physics broad-spectrum (UV radiation) and chemical (xenobiotics) agents that are able to change the skin structure and function [1].One sign of damaged skin (aging) is the loss of elasticity and flexibility of the skin that causes wrinkles. Wrinkles on the skin not only occurred because of extrinsic factors but also intrinsic factors, there are genetic that influencing individually and cumulative damage of genes and proteins environmental[2]. As person get older, the skin hyaluronic acid concentration is reduced, so that the skin becomes loose, pale and dull [3,4]. Hyaluronic acid greatly influences the hydration and extracellular matrix-forming molecules. Hyaluronic acid may also interact with receptors that activate multiple lines for cell migration, proliferation, and gene expression [5]. Hyaluronic acid content can be influenced by a variety of compounds, for example is hyaluronidase contained in the dermis of the skin[6].

Hyaluronidase is mucopolysaccharides which hydrolyseglycosaminoglycans in the extracellular matrix during tissue restoration, this process including hyaluronic acid. When hyaluronic acid decreases under conditions in which the activity of hyaluronidase increases, skin humidity and elasticity reduced. Thus, inhibitors of hyaluronidase are useful for cosmetic ingredients because they have anti-wrinkle and anti-aging effect for the skin. Based on it, a compound that can inhibit the wrinkling of the skin is required. Polyphenols are natural compounds from plants which are widely studied because it has the effect of antioxidants that may prevent skin damage caused by UV exposure. One of the polyphenolic compounds that have high antioxidant activity is phenol[7].

Alpiniazerumbet is one of the plants in Indonesia that can be used as a deterrent for skin damage (anti-wrinkle), it is a plant that grows widely in tropical and subtropical regions [8]. *Alpiniazerumbet* rhizome has a polyphenol content in the form of 5,6-dyhydrokawain (DK), dihydro-5,6-dyhydrokawain (DDK), 12-labdadiene-15[8-10]. Chompoo, et al. research in Japan, found that *Alpiniazerumbet* rhizome phenolics level that extracted using ethanol was 38.50±0.47 mg/g extract and antioxidant value (IC_{50}) was 145.07±1.68 mg / mL [11].

Researchers intended to develop the *Alpiniazerumbet* rhizome into an extract and then prepared into the form of nanoparticles in order to make it easier for the active ingredients to penetrate into the dermis of the skin [12,13]. Preferred nanoparticle delivery system is Solid Lipid Nanoparticles (SLN). Application of nanotechnology in the pharmaceutical field has many advantages which are to improve solubility, doses reduction and absorption increase. SLN using physiological lipid that is solid phase at room temperature and surfactant in emulsification, high drug loading is potential to improve the performance of pharmaceutical products and large applicative spectrum (dermal, oral, iv), also has a better stability compared to liposomes [14].

2. Material and Method

2.1 Materials

Rhizome of *Alpiniazerumbet* was purchased from Bogor, West Java (Indonesia). Hyaluronic acid (H5542), bovine serum albumin (A4503) and hyaluronidase from bovine testes (IS-(H3506) were purchased from Sigma-Aldrich (USA). Sodium chloride, Sodium acetate and hydrochloride acid were purchased from Merck (Germany). Polyethyleneglycol-8 beeswax was from Gattefosse (France). Acrylylglucoside was from BASF (Germany).

Instrument
Particle Size Analyzer Zetasizer (Malvern), Stirrer (IKA, RW-20), Ultraturrax (IKA, T25), Sonicator (Qsonica, Q700), ELISA reader (Multiskan Go Reader, Thermo Fisher Scientific TM Multiscan, N12391), heater (IKA , C-MAG HS7), analytical balance (Mettler, toledo), Viscometer (Brookfield DV II + Pro), pH meter (Methorm, 620), Incubator (ESCO), oven (Memmert), centrifugator (KOKUSAN, H103-N) , water bath (Memmert), TEM (JEOL, JEM-1400), rotator (EnduroTM MiniMix), vortex (Maxi Mix® II, M37600).

2.2 Method

Determination of the plant
Alpiniazerumbet plants were determined at Indonesian Institute of Sciences, Research Center for Biology, West-Java, Indonesia.

Preparation of Extract
*Alpinia zerumbet*rhizome extract was prepared using rhizome powder with 70% ethanol as an extractor solution. Afterwards the mixture was extractedusing maceration kinetics method. The filtrate was filtered and evaporated using a vacuum rotary evaporator at a temperature of ±50°C, pressure of 100 mmHg, and a speed of 70 rpm to have an extract viscous[12].

Characterization of extract
Characterization of the extract including: organoleptic, pH and solubility with some solvent. Solubility tested by dissolving 1 gram of extract with 10 mL of distilled water, propylene glycol, and 70% ethanol [13].

Anti-hyaluronidase Activities of extract
Anti-hyaluronidase activity of *Alpiniazerumbet* rhizome extract was tested using *in vitro* method. A stock solution of *Alpiniazerumbet* rhizome extract was prepared by dissolving 20 mg of rhizome extract in 100% DMSO. Then, *Alpiniazerumbet* rhizome extract working solution was prepared by diluting stock solution until reach the concentration of 2000µg/mL. Furthermore, extract solution was processed in the vortex and diluted with 10% DMSO until the working solution concentration reach 1000, 500, 250, 125, and 62.5 µg/mL.

a) Anti-hyaluronidase reagent preparation
Including 300mM phosphate buffer, 0.3% w/v hyaluronic acid solution, enzyme diluent (Dissolve 2.4 mg/ml sodium phosphate monobasic, containing 4.5 mg/ml of sodium chloride and 0.1 mg/ml BSA in distilled water. Adjust pH until 7, at 37°C), Albumin acid solution (Dissolve 3.27 mg/ml sodium acetate in distilled water, containing 4.5 µl/ml acetic acid and 1 mg/ml BSA adjust the pH until 3.75, 0,02 mg hyaluronidase enzyme in 1 ml of enzyme diluent.

b) Anti-hyaluronidase activity test
Anti-hyaluronidase activity was measured based on a method that has been described by Sigma-Aldrich and Tu and Tawata (2015) with slight modifications. 25 µL samples (0-166.67 µg/mL) was pre-incubated at 37°C for 10 minutes with 3 µL hyaluronidase from bovine testes type I-S (Sigma H3506) (1U/mL in 20mM phosphate buffer, pH 7 containing 77mM sodium chloride, and 0.01% bovine serum albumin), and 12 µL of phosphate buffer (300mM, pH 5.35) at 37°C for 10 minutes. After pre-incubation, 10 µL of hyaluronic acid substrates (0.03% in 300mM phosphate buffer, pH 5.35) (Sigma H5542), then re-incubated at 37°C for 45 minutes. Hyaluronic acid decomposition reaction was stopped by adding 100 mL of albumin acidic acid (24 mM sodium acetate, 79 mM acetic acid, and 0.1% BSA). The solution mixture placed in room temperature for 10 minutes. The absorbance of the colored product was measured at 600 nm wavelength [15,16].

c) The percentage of anti-hyaluronidase activity.
Anti-hyaluronidase activity was determined by measuring the absorbance using ELISA reader at 600 nm wavelength. From the absorbance measurement, percentage of anti-hyaluronidase activity can be calculated according to the following formula:
B: Absorbance without the enzyme activity
S: Absorbance of enzyme activity with sample addition
IC_{50} was calculated using linear regression of absorbance, sample concentration (in logarithms) as the x-axis and percent inhibition as the y axis. From the equation y = a + bx, IC_{50} can be calculated.

$$\% \text{ Anti-hyaluronidase activity} = \frac{S}{B} \times 100\%$$

Preparation of extract loaded SLN
Alpiniazerumbet rhizome extract loaded SLN was conducted using hot speed homogenization -. ultrasonication. Polyethyleneglycol-8 beeswax was melted above water-bath at 60°C, then rhizome extract that has been dissolved in propylene glycol was added to the melted polyethyleneglycol 8-beeswax. Acrylylglucoside and distilled water were heated at 60°C. Polyethyleneglycol 8-beeswax and extract mixture were poured into mixture of distilled water and acrylylglucoside, then mixed by stirring using stirrer at a speed of 500 rpm. Then stirred again using ultraturrax at a speed of 20,000 rpm for 10 minutes, then cooled at 2-5^{o}C temperature while stirring using ultraturrax with 5,000 rpm. Furthermore, ultrasonication was conducted at 50 amplitude for 10 minutes. Extract loaded SLN was characterized[17].

Characterization of extract loaded SLN [18]

a) Particle size and polydispersity index
Particle size and polydispersity index was measured based on the population distribution of the nanoparticles. Small sample of extract loaded SLN were poured into the

container. Afterwards, the sample was diluted and analyzed using Particle Size Analyzer-Zetasizer (Malvern, UK).

b) Zeta potential

Small samples (100µL) of extract SLN aliquot (fraction) were dispersed in 50.0 mL distilled water. Then the mixture was measured using a Particle Size Analyzer-Zetasizer (Malvern, UK).

c) Morphological analysis

Extract loaded SLN morphology analysis was performed by using Transmission Electron Microscopy (JEOL JEM 1400). Extract loaded SLN sample was diluted with a ratio of 1:10. Then dripped onto Cu substrated grid and wait until dry. Then, added some drops of 2% uranyl acetate solution, wait until dry and analyzed using the 100KV TEM with various magnifications.

3. Results and Discussion

Characterization of the extract

Characterization of *Alpiniazerumbet* rhizome extract which has brown color, distinctive fragrance of *Alpiniazerumbet* rhizomes with pH of 5.65. Easily dissolved in distilled water and propylene glycol (1:10) and dissolved in 70% ethanol (1:20).Solvent associated with the extract loaded SLN preparation process and anti-hyaluronidase activity in vitro test.

Anti-hyaluronidase activity of the extract

Anti-hyaluronidase activity of *Alpiniazerumbet* rhizome extract usingturbidimetric method (% transmitter at 600 nm wavelength, 1 cm light path). This method is used by measuring hyaluronic acid fragments formed by the decomposition of hyaluronic acid by hyaluronidase. Once albumin acid solution added, samples containing small fragments of hyaluronic acid will be more turbid, while the sample thatisn't inhibited will be more clear. Anti-hyaluronidase activity test was measured based on a method that has been described by Sigma Aldrich (2015) with slight modifications. Preparation of solution mixture that are used listed in Table 1.

Table 1: Total solution of anti-hyaluronidase activity test

material	*Well (microtiter plate)*		
	Control (µl)	Sample (µl)	Blank sample (µl)
Hyaluronidase	3	3	-
Phosphate buffer	37	12	15
Samples	-	25	25

In Table 1, the materials which has been preparedsequentlyincubated at 37°C for 10 minutes, each well of 96 well-microtiter plate was added 10 µLhyaluronic acid substrate, then re-incubated at 37°C for 45 minutes and added 100 µL of acidic albumin solution to stop the reaction. Before measuring the absorbance, 96 well-microtiter plate that has been filled by mixturedand acidic albumin solution, put at room temperature for 10 minutes. The absorbance was measured using ELISA reader with 600nm wavelength. Anti-hyaluronidase activity of *Alpinia zerumbet*rhizome extract listed in Figure 1.

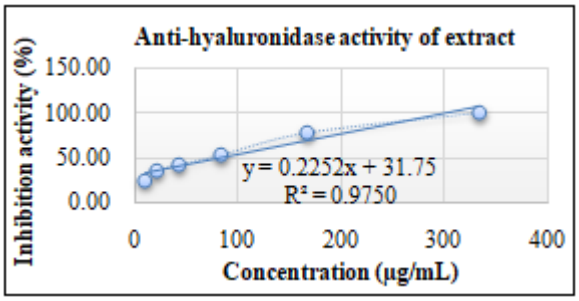

Figure 1: Concentration of *Alpinia zerumbet*rhizome extract Vs inhibition anti-hyaluronidase activity

IC_{50} of Anti-hyaluronidase activity test that has been doneis 81.04µg/mL. Hyaluronidase is an enzyme that degrades hyaluronic acid, causing wrinkles on the skin, because it reducewater content of the skin. Inhibition of hyaluronidase is one way to prevent wrinkles on the skin, because it can inhibit the degradation of water content. In vitro test was conducted by measuringhyaluronic acid fragments which are the result thatformed because the decomposition of hyaluronic acid by hyaluronidase.Once albumin acid solution added, samples containing small fragments of hyaluronic acid will be more turbid, thenabsorbance can be measuredwith colorimetric method by using ELISA reader at 600 nm wavelength.

Characterization of extract loaded SLN

Characterization of *Alpiniazerumbet*rhizome extractloaded SLN including particle size, polydispersity index, zeta potential, and morphology of nanoparticles using Transmission Electron Microscopy (JEOL JEM 1400).

The particle size and polydispersity index of extract loaded SLN

The particle size and polydispersity index of*Alpiniazerumbet* rhizomeextract loaded SLNare listed in Table 2 and Figure 2.

Table 2: Particle size and polydispersityindex of extract loaded SLN

Particle size (nm)	Average particle size (nm)	polydispersity index	Average polydispersity index
138.3	138.6	0.227	0.226
138.5		0.233	
139.0		0.218	

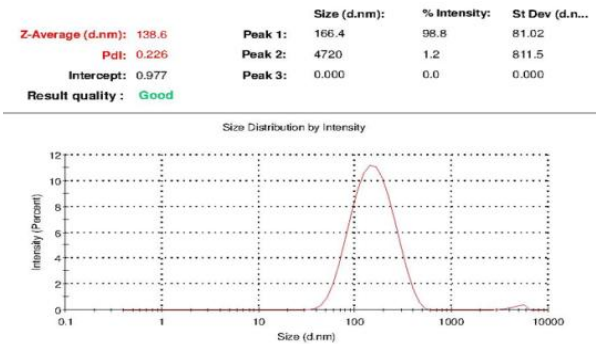

Figure 2: Particle size and polydispersity index of extract loaded SLN

In Table 2 and Figure 2,*Alpiniazerumbet* rhizome extract loaded SLN particle size is 138.6 nm. Small particle size

ofextract loaded SLN is the result of using high speed homogenization-ultrasonication method. High shearing force and waveable to break down particles into smaller size. Nanoparticles with sub-micron size provides relatively high intracellular uptake compared with microparticles and having greater range of availability in the biological targets, thus relatively easierto distribute. With particle size of 138.6 nm extract loaded SLN are expected to release the active substance into the skin more quickly and distributed evenly into the intracellular tissue. The result of polydispersity index was 0.226. Polydispersity index shows the uniformity of nanoparticles size. Extract loaded SLN polydispersity index close to zero, which means the size of the nanoparticles are uniform.

Zeta potential of extract loaded SLN

Zeta potential of *Alpiniazerumbet*rhizome extract loaded SLN are listed in Table 3 and Figure 3.

Table 3: Zeta potential of extract loaded SLN

Zeta potential (mV)	The average zeta potential (mV)
-30.7	-29.9
-30.0	
-28.9	

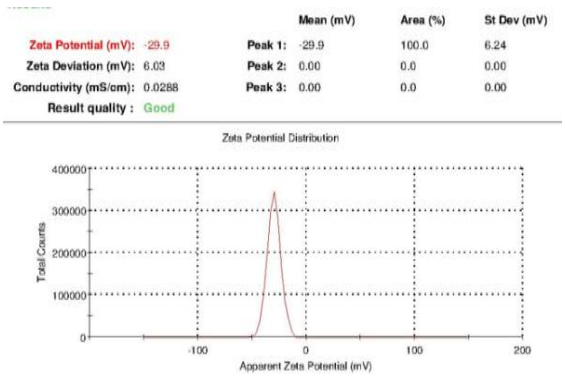

Figure 3: Zeta potential of extract loaded SLN

In Table 3 and Figure 3, *Alpiniazerumbet*rhizome extract loaded SLN zeta potential is -29.9 mV. Zeta potential is a measure of the repulsive force generated between the particles. Zeta potential of nanoparticles determine colloidal system which is stabilized by electrostatic forces. If all of the particles in colloidal systems have more positive zeta potential than +30 mV or more negative than -30 mV, then it can be declared stable. Because, each particle have high repulsion, so there is no tendency for particles to aggregate. According to the DLVO theory, colloidal stability of emulsion depends on particlesrepulsion (electric double layer style) to counter particles attraction (Van der Waals force), which can cause irreversible aggregation. It is expected that the colloidal system in *Alpiniazerumbet*rhizome extract SLN uniformly dispersed and stabilized.

The morphology of extract loadedSLN

*Transmission Electron Microscopy*is a technique to determine the shape and morphology of lipid nanoparticles. *Alpiniazerumbet* rhizome extract loaded SLN morphological analysis results listed in Figure 4.

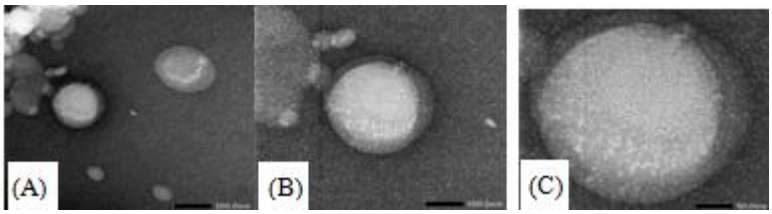

Figure 4: Transmission Electron Microscopy photograph of*Alpiniazerumbet*rhizome extract loaded SLN, (A) scale of 200 nm with 20,000 x magnification, (B) scale of 100 nm with 40,000 x magnification, (C) scale of 50 nm with 80,000 x magnification

*Alpiniazerumbet*rhizome extract loaded SLN morphological analysis results usingTEM (Figure 4) shows that the extract loaded SLN globules have it size below 200 nm with spherical shape. The image shown that extract loaded SLN forming a solid form, distributed evenly and fairly uniform, this result confirmed by polydispersity index test result (<1).

4. Conclusion

*Alpiniazerumbet*rhizome extract in 70% ethanol have anti-hyaluronidase activity with IC_{50}value of 81.04µg/mL. *Alpiniazerumbet* rhizome extractloaded SLN meet the characterization requirements of nanoparticles with 138.6 nm particle size, 0.226 polydispersity index,and -29.9 mV zeta potential with spherical morphology.

5. Acknowlwdgment

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