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# Antithrombotic and Antioxidant Activities of Binahong [*Anredera cordifolia* (Ten.) Steenis] Leaf Ethanol Extract and its Nanoemulsion Preparation in Swiss Webster Mice

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## Abstract

Platelet aggregation has the potential to form thrombi and result in cardiovascular system diseases such as myocardial infarction and ischemic stroke - one of the leading causes of death globally. Traditionally, people use binahong leaves as blood thinners. Therefore, this study aims to obtain scientific data on the efficacy of binahong leaf as antithrombotic. The ethanol extract of binahong leaves was obtained from PT Phytochemindo Reksa with product code 1A12E50 batch number 024CV. The characteristics of the extract were determined using WHO guidelines and the Indonesian herbal Pharmacopoeia which included phytochemical screening, determination of water content, water soluble extract content, ethanol soluble extract content, total ash content, and acid insoluble ash content. The efficacy test was carried out on male Swiss-Webster mice with antithrombotic parameters which include bleeding time, coagulation time, platelet count, and platelet aggregation inhibition test, and antioxidant activity through lipid peroxidation, DPPH test, and determination of nitric oxide levels. The three doses of binahong leaf extract tested were 50 (BLEE50), 100 (BLEE100), and 200 mg/kg bw (BLEE200) which were administered orally once a day for 14 consecutive days. The results showed that BLEE50 and BLEE100 could increase bleeding time on H7 (7.61±1.79% and 3.72±1.76% vs 1.08±0.90%) and H14 (13.81±4.42% and 5.06±2.30% vs 1.66±1.09%) and coagulation time at H7 (5.01±1.36% and 4.18±1.67% vs 1.38±1.08%) and at H14 (7.92±1.97% and 7.19±1.96% vs 1.70±1.10%) significantly ($p<0.05$). The two doses of BLEE were formulated in the form of nanoemulsions with the Self Nano Emulsifying Drug Delivery System (NBLEE50 and NBLEE100) were also able to prolong bleeding time and coagulation time significantly ($p<0.01$) but only NBLEE50 prolonged bleeding time ($p<0.05$) significantly against BLEE50. In the test of the anti-platelet aggregation effect with ADP as an inducer, both doses of BLEE and the nanoemulsion preparation

(NBLEE) could significantly ($p<0.01$) inhibit platelet aggregation with a percentage of inhibition >70% which was not different from the standard (acetylsalicylic acid). In the antioxidant effect test using the DPPH method, BLEE has an $IC_{50}$ = 66.08µg/mL which is classified as a strong antioxidant. Both doses of BLEE and its NBLEE could significantly ($p<0.01$) inhibit lipid peroxidation in plasma and liver and NO radicals formation. BLEE50 can significantly ($p<0.05$) reduce mean platelet volume (6.05±0.24fL vs 6.55±0.34fL) and platelet distribution width (8.52±0.36% vs 9.25±0.42%). Based on those results, BLEE has the potential to be used as an antiplatelet aggregation and antioxidant.

**Keywords:** *Anredera cordifolia* leaves, nanoemulsion, antithrombotic and antioxidant activities

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## 1 Introduction

Today, cardiovascular system-related diseases are the leading cause of death in the world. Stroke, myocardial infarction, and heart attack are the main causes of morbidity and mortality associated with cardiovascular disease [1]. The main problem is the formation of a thrombus that can block blood flow. In the guidelines for stroke treatment, there are two most used drugs, namely acetylsalicylic acid (aspirin) and clopidogrel to prevent the formation of blood clots. Although clinically beneficial, the side effects of both drugs such as the reluctance by acetyl salicylic acid, muscle pain, rash, and allergies to clopidogrel, are still not overcome. Therefore, it is necessary to look for safer alternative medicines [2].

The potential of nature in Indonesia which is rich in natural resources is one of the supporting factors for researchers to develop medicine from natural materials of plant origin. One plant that is traditionally widely used as a blood thinner in anticipation of stroke is the leaves of *Anredera cordifolia*. *Anredera cordifolia* with full Latin name [*Anredera cordifolia* (Ten.) Steenis] and the name of the regional synonym in Indonesia is Binahong, in China known as Dheng San Chi, in Europe named heartleaf madeiravine, in South America known as madeira-vine in Australia it is called "madeira vine"; in Argentina, it is known as "zarza-parilla" and "papa santa". The leaves and tubers are also used as a source of protein [3] [4], [5]. In Indonesia, the leaves are fed directly as "lalapan". The roots and all parts of the above-ground plant are efficacious and have been consumed for thousands of years by the Chinese, Koreans, Taiwanese, and Indonesians [3].

Pharmacologically, many researchers have studied its pharmacological properties [6], [7]. Some pharmacological activities that have been studied [6] as antibacterial, antifungal, and may lower tumor titers necrosis factor and other inflammatory mediators in macrophage lines [8] and as an antiaging effect [6]. In vivo, binahong leaf extract (*Anredera cordifolia*) is efficacious as analgetic and anti-inflammatory, lowering rat uric acid levels [7], improves the immunity of guinea pig test animals before and after childbirth [9], heal wounds on guinea pigs [10], lower blood pressure in rabbits and toads [7], has diuresis and blood pressure-lowering effects in mice, protecting against damage to the renal tubules of mice Sprague Dawley [11], treating kidney failure in Wistar mice [5], antiobesity, lowering total cholesterol, triglyceride levels, and potentially as an anti-cancer [6]. *Anredera cordifolia* rhizome can also inhibit trypsin and stimulate nitric oxide formation [7].

Phytochemical filtering indicates the presence of saponins, steroids/terpenoids, flavonoids, alkaloids, tannins in *Anredera*

*cordifolia* [2], [9], [10]. We have found flavonoids and polyphenols responsible for the antiaggregating effects of platelets. Due to its flavonoids and polyphenols content, we focused this study on antithrombotic effect test, lipid peroxidation test (MDA levels), and antioxidant activity in *Anredera cordifolia* ethanol extract in which the studies are still very limited. Because the dosage form affects the bioavailability of the drug and its biological response, the dosage form in this study wasmade in nanoemulsion preparation containing *Anredera cordifolia* leaves ethanol extract which then used in the antithrombotic test. Antioxidant activitywere determined in vitro by the DPPH method and in vivo by lipid peroxidation inhibition tests (determination of MDA levels) [2], [12], [13], [14] and NO level [15].

## 2 Materials and Methods

### 2.1 Materials

The ingredients used include: Binahong (*Anredera cordifolia*) leaf ethanol extract (Phytochemindo Reksa®), ammonia, chloroform (Merck®), toluene (Merck®), amyl alcohol, HCl, NaOH, $FeCl_3$, ether, dragendorf reagent, steasny reagent, gelatin, Lieberman burchard reagent, 96% ethanol (Merck®), sodium citrics, acetylsalicylic acid/acetosal/aspirin, sodium chloride 0.9%, Tween 80, PEG 400, virgin coconut oil (VCO), phosphate buffer, adenosine diphosphate/ADP (Sigma-Aldrich®), absolute ethanol (Merck®), methanol p.a (Merck®), trichloroacetic acid, tiobarbiturate acid (Merck®), diphenyl picrylhydrazyl/DPPH (Merck®).

### 2.2 Characterization of *Anredera cordifolia* Leaf Extract

Ethanol extract of binahong leaves is obtained from PT. Phytochemindo Reksa with product code 1A12E50 batch number 024CV on August 21, 2021. The characterization of extracts is carried out according to WHO guidelines and Indonesian Herbal Pharmacopoeia (2017) which include phytochemical filtering, determination of moisture content, water soluble juice content, ethanol soluble juice content, total ash content, and acid insoluble ash levels [16], [17].

### 2.3 Experimental Animals

The test animal used in this study was the Swiss-Webster mice with body weight range from 25-30 grams, aged 2-3 months, obtained from the School of Life Technology Sciences, Bandung Institute of Technology. Animals were placed in transparent cages with sawdust cage mats, kept in a room with a temperature of 24-28 °C, humidity (Rh) of 60-70 in accordance with the rules of Animals Used in Biomedical Research (2008). Animal feed is obtained from the PT Bio Rat.

### 2.4 DPPH Radical Scavenging Assay

The ability of ethanol extract of binahong leaves (BLEE) toward free radical was tested using the appropriate DPPH (diphenyl picrylhydrazyl) radicals as performed by [18], [19], [20] with modification. A total of 1 mL of BLEE with various concentrations (10, 40, 70, and 100 ppm) was added to the 2 mL DPPH 0.1 mM. The mixture then shaken and incubated at room temperature for 30 minutes in a dark place. This solution absorbance then measured at maximum wavelength ($\lambda_{max}$) of 516 nm. The same treatment was also done for blank solution (DPPH solution that did not contain test ingredients) and standard vitamin C (ascorbic acid) with concentrations of 1, 2, 3, 4, and 5 ppm. $\lambda_{max}$ used for ascorbic acid was 515 nm. The blank solution consists of 2 mL DPPH 0.1 mM and 1 mL methanol p.a. Data from the measurement of absorbance dianalisis percentage inhibition/antioxidant activity using the equation below. Calibration curve plotted with % DPPH scavenged versus standard antioxidant concentration (Vitamin C) [18], [19], [20].

$$\%DPPH\ Scavenged = \frac{A_{blank} - A_{Sample}}{A_{blank}} \times 100\%$$

Information:
Ablank = Blank absorbance value
Asample = Sample absorbance value

### 2.5 Formulation of Nanoemulsion of BLEE

Nanoemulsion of BLEE (NBLEE) was formulated by mixing BLEE into the oil phase using virgin coconut oil (VCO). To the mixture then added surfactants and co-surfactant namely Tween and PEG 400 while heated at a temperature of 60 °C and stirred at a speed of 600 rpm using magnetic stirrer for 15 minutes. The oil and surfactant phase mixture were stirred back using ultraturrax at 10,000 rpm for 4 minutes. Furthermore, the solution was mixed with aquades as a water phase, sonicated using a waterbath sonicator for 30 minutes at frequency of 59 kHz and a strength intensity of 100%. The preparation formula was selected based on droplet size tests, polydispersity indexes, and stability tests. The molecular diameter and polydispersity index of the preparation were measured with the Particle Size Analyzer (PSA) and the stability of the nanoemulsion preparation was determined through mechanical and physical stability tests. Mechanical stability was carried out by centrifugation of the preparation in the centriphant tube at a speed of 6000 rpm for 30 minutes. While the physical stability test uses the stability method accelerated by the freeze thawing method, namely by storing the preparations in temperatures of -4 °C, 25 °C (room temperature), and 37 °C (stored in a climatic chamber with an rH of 15%), each for 24 hours [14], [21], [22].

### 2.6 Antithrombotic test

Antithrombotic tests were divided into two stages, namely the dose orientation test and the actual test. The orientation test aims to determine the test dose to be formulated in the form of nanoemulsion for further testing. At the orientation stage, 3 doses of BLEE are used, i.e., 50, 100, and 200 mg/kg of test animal body weight. Animals were grouped randomly, and each group consists of 5 animals. Test provision group, comparison (acetylsalicylic acid; 42.25 mg/kg bb) and water as a control were administered orally 1× daily for 14 consecutive days. Bleeding time and coagulation time were determined on days 0, 7, and 14. Orientation results were statistically analyzed to determine the next test dose [2], [23].

Two doses of extract in further testing, both in extract form (BLEE) and nanosemulsion dosage-form (NBLEE) were carried out in advanced antithrombotic testing. Grouping of animals and the administration of preparations are carried out orally as in the orientation test stage. Measurement of antithrombotic test parameters were carried out on the 14th day after administration of BLEE and NBLEE. At the end point of the test, blood was drawn through cardiac puncture after the animal was humanely sacrificed using carbon dioxide gas. Mice blood was accommodated in a tube containing a 3.18% sodium citrate solution, then centrifuged and the plasma separated for platelet aggregation testing. Liver and brain were isolated for antioxidant activity assays [2], [23].

### 2.7 Bleeding time measurement

The tip of the squeaked tail was injured, the blood that comes out is then absorbed on the depositing paper every 30 seconds. The time interval between the onset of the first drop of blood until the blood stops flowing is the time of bleeding [23], [24].

### 2.8 Coagulation time measurement

The tip of the tail squeak was injured, the outgoing blood was inserted into the non-heparinized capillaries. Capillary pipes were broken every 15-second interval until the formation of fibrin threads is observed on the broken part. The start time of blood discharge until fibrin threads form is the time of coagulation [23], [24].

### 2.9 Inhibition of platelet aggregation

250 µL of blood plasma was added with 30 µL sodium chloride 0.9% b/v. Plasma absorption was measured using a spectrophotometer at a wavelength of 600 nm. Then to the plasma mixture 30 µL ADP solution 5 µM was added as an inducer of platelet aggregation then incubated for 20 minutes in a shake incubator with a temperature of 37°C and then the absorption was re-measured. Next, the difference in plasma absorption before and after the administration of the inducting solution was calculated. The magnitude of the decrease in plasma uptake compared to normal controls

determined the inhibition degree and it statistical significance was determined using SPSS application version 25 [22], [24].

### 2.10 Platelet count measurement

The blood profile was determined using a hematology analyser. Squeaky blood was taken with cardiac puncture, inserted into a special capillary pipe, and then inserted into a hematology analyzer that has been connected with blood thinning reagents. Furthermore, all blood parameters consisting of the number of erythrocytes, leukocytes, platelets, HCV, MCV, and MCHC were recorded [2], [25].

### 2.11 Lipid Peroxydation

Animals were grouped and treated as in the antithrombotic test. Lipid peroxidation was tested using plasma, liver, and brain. For plasma sampel, into 250 µL plasma is added 250 µL TCA and 500 µL TBA. After shaking until homogeneous, it was heated over a waterbath temperature of 95-100 °C for 10 minutes. After the mixture was cold, it was then centrifuged at 4000 rpm for 10 minutes. The acquired supernatants were then separated and its absorption was measured at 532 nm. For liver and brain samples, each was taken as much as 100 mg of organs. Homogenate was made in phosphate buffer saline at pH 7.4 with a sample:buffer ratio of 1:1 or 10% by rubbing using mortar and pestle in the cold temperature. Furthermore, the centrifugation process was carried out at 3000 rpm for 10 minutes. A total of 0.5 mL of supernatant was added with 0.5 mL of TCA 20% and 1 mL of TBA 0.67%, then shaken to obtain homogeneous sample, then heated at a temperature of 95-100 °C in a water bath for 10 minutes. After cooling, it was centrifuged at 4000 rpm for 10 minutes. The supernatant obtained was further cooled under the water and measured its absorption at a wavelength of 532 nm [26].

### 2.12 Determination of NO level

In order to determine NO level, we used the animals that have been treated as in the lipid peroxidation test. Their total blood was taken at the end point and the plasma was separated. A total of 200 µL of plasma was oxidized with 200 µL of potassium permanganate for 10 minutes, then 800 µL of 5% salicylic acid was added in concentrated sulfuric acid and allowed to stand for 20 minutes. Next, up to 10 mL of 8N sodium hydroxide was added in a volumetric flask then centrifuged. Absorption was measured using a UV-VIS spectrophotometer at a wavelength of 414 nm. The standard used is sodium nitrite.

## 3 Results and Discussion

### 3.1 Extract characterization

The dried leaves extract of binahong (BLEE) used in this study was grayish-green powders with a distinctive smell and taste. The yield of extract obtained is 50% with ethanol as a solvent. BLEE used in this study met the requirements of the Indonesian Herbal Pharmacopoeia for moisture content, total ash content, acid insoluble ash, and water-soluble juice content but not for ethanol soluble juice levels. This data shows that the soil where it grows has an effect on the chemical content of BLEE [3], [5], [16].

Table 1. Characteristics of BLEE

| Parameters | Result | References |
|---|---|---|
| Moisture content (% v/w) | 6,9 | >5 |
| Total Ash Content (% w/w) | 16,3 | <16,3 |
| Acid insoluble Ash Levels (% w/w) | 1,8 | <1,9 |
| Water soluble content (% w/w) | 89,6 | >13,5 |
| Ethanol soluble content (% w/w) | 11,2 | >19,6 |
| Cehmical content: | | |
| a. Flavonoid | + | + [4] |
| b. Alkaloid | - | - [5], [7] |
| c. Tannin | - | - [5], [7] |
| d. Saponin | + | + [27] |
| e. Steroid | + | + [27] |
| f. Triterpenoid | + | + [27] |
| g. Quinone | - | - [27] |

The results of phytochemical screening on the extract showed that BLEE contained flavonoids, sapoins, steroids, and triterpenoids as can be seen in Table 1. This result is in accordance with the findings of some previous researchers.

### 3.2 Antioxidant test with DPPH method

Standard curve data, average absorbance, and percentage of inhibition (%) of ascorbic acid can be seen in Table 2. Ascorbic acid

compounds used as antioxide standards obtained a 50% inhibition concentration ($IC_{50}$) of 4.82 µg/mL. Antioxidant activity test with DPPH method was done to get an idea of the presence of compounds in the ethanol extract of *Anredera cordifolia* leaves that can capture free radicals [19]. The value of antioxidants is expressed by $IC_{50}$ (Inhibitory Concentration) which is defined as the magnitude of the concentration of a single test that can reduce free radicals by as much as 50%. The smaller the $IC_{50}$ value, the higher the free radical damping activity. As a standard, vitamin C or ascorbic acid is used as a reference to see antioxidant activity.

Table 2. Absorbance of a standard

| Concentration (ppm) | Absorbance | | | Absorbance Average | Inhibition (%) |
|---|---|---|---|---|---|
| | Abs. 1 | Abs. 2 | Abs. 3 | | |
| 1 | 0,760 | 0,768 | 0,763 | 0,763±0,004 | 1,68 |
| 2 | 0,680 | 0,676 | 0,669 | 0,675±0,006 | 13,02 |
| 3 | 0,577 | 0,566 | 0,560 | 0,568±0,009 | 26,80 |
| 4 | 0,480 | 0,477 | 0,473 | 0,477±0,004 | 38,53 |
| 5 | 0,369 | 0,360 | 0,364 | 0,364±0,004 | 53,09 |
| Standard | 0,706 | | | | |

$y = bx + a$ $\quad y = 12{,}8349\ x - 11{,}8815$

$50 = 12{,}8349\ x - 11{,}8815$ $\quad IC_{50} = \frac{50+11{,}8815}{12{,}8349} = 4{,}82\ \mu g/mL$

Based on absorbance data and percentage of inhibition (%) of BLEE with DPPH method that can be seen in Table 3, it is shown that ethanol extract of binahong leaves have a fairly high antioxidant activity characterized by a small $IC_{50}$ value of 66.08 µg/mL. $IC_{50}$ values that are in the range of 50-100 belong to powerful antioxidants [19].

Table 3. Absorbance data and % Inhibition of BLEE

| Concentration (ppm) | Absorbance | | | Absorbance Average | % Inhibition |
|---|---|---|---|---|---|
| | Abs. 1 | Abs. 2 | Abs. 3 | | |
| 10 | 0,533 | 0,537 | 0,538 | 0,536 | 24,08 |
| 40 | 0,424 | 0,421 | 0,425 | 0,423 | 40,08 |
| 70 | 0,342 | 0,343 | 0,345 | 0,343 | 51,42 |
| 100 | 0,248 | 0,250 | 0,251 | 0,249 | 64,73 |

$y = bx + a$ $\quad y = 0{,}4443\ x + 20{,}641$

$50 = 0{,}4443\ x + 20{,}641$ $\quad IC_{50} = \frac{50\ -\ 20{,}641}{0{,}4443} = 66{,}08\ \mu g/mL$

### 3.3 Antithrombotic effect of BLEE (Preliminary)

At the orientation stage, after giving the test extract, there was a significant increase in bleeding time (Figure 1) after 7 and 14 days of giving the test extract at doses of 50 (BLEE50) and 100 mg/kg body weight (BLEE100). Blood coagulation time also increased significantly ($p<0.01$) in the group given BLEE50 and BLEE100 on the 7th day and the BLEE200 on the 14th day (Figure 2). Bleeding time and coagulation time also increased significantly ($p<0.01$) after administration of acetylsalicylic acid which was used as the standard in this study. Based on this orientation phase data, and for efficiency, two extract doses, *i.e.,* BLEE50 and BLEE100, were selected for further investigation and formulated in the form of nanoemulsions (NBLEE50 and NBLEE100).

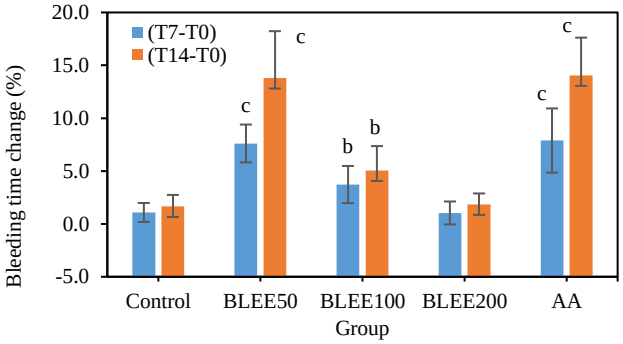


Figure 1 Bleeding time change during treatmen with binahong leave etanol extract (BLEE). b=p<0.05; c=p<0.01 compared to than of control; AA = Acetyl salicylic acid (standard)

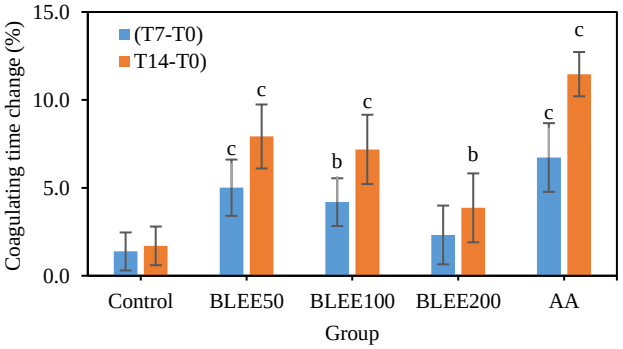


Figure 2. Coagulating time changes during treatment with binahong ethanol extract (BLEE). b=p<0.05; c=p<0.01 compared to than of control: AA = Acetyl salicylic acid (standard)

### 3.4 Nanoemulsion preparations of BLEE

Binahong leaf extract nanoemulsion (NBLEE) was formulated using virgin coconut oil (VCO) as the oil phase, Tween 80 as surfacfigretant, and PEG 400 as co-surfactant. The formula and evaluation results of nanoparticle diameter and polydispersity index can be seen in Table 4.

Table 4. Optimization of NBLEE formula

| Formulation | F1 | F2 | F3 | F4 |
|---|---|---|---|---|
| EEAL | 50 | 50 | 50 | 50 |
| VCO (mL) | 2 | 2 | 1 | 1 |
| Tween (mL) | 1,5 | 1 | 1,5 | 1 |
| PEG 400 (mL) | 1 | 1 | 1 | 1 |
| Aquadest (mL) | 5,5 | 6 | 6,5 | 7 |
| Diameter (nm) | 171,1 | 1692,7 | 113,3 | 175,6 |
| Polydispersity Index | 0.296 | 0.607 | 0.225 | 0.298 |

In some of the formulas tested, one of the most stable formulas has been obtained which had the best diameter and polydispersity index compared to others. Formula F3 and F4 had the most stable performance compared to the other two formulas (Figure 3). This can be seen in F3 and F4 which have a diameter of < 200 nm and smaller polydisperstas index values of 0.225 and 0.298 compared to other formulas (Table 4). So that the F3 and F4 formulas were then carried out for physical and mechanical stability testing. A good particle size of nanoemulsion has size ranged from 20-200 nm. As small as the particle size of an active substance in the SNEDDS (Self-Nanoemulsifying Drug Delivery System) preparation, it will further increase its stability and distribution in the dissolved media [14], [21], [28], [29].

The stability test of NBLEE were carried out using the freeze-thawing method to see the effect of temperature and storage time on nanoemulsion phase separation [14], [30]. It showed that only F3 was stable which did not undergo separation. Formula F4 appeared to experience creaming on the surface of the preparation, while formula F3 did not show any creaming or phase separation. The stability of this nanoemulation occurs because of the ability of surfactants and cosurfactants to reduce the surface tension of the emulsion between the oil phase and the water phase. The greater the ability of surfactants and cosurfactants to reduce interfacial tension, the more stable a nanoemulation preparation will be [14], [31]. The process of decreasing the surface tension helps to produce the globule size of the formed emulsion, the use of cosurfactants can improve the performance of the surfactant in an effort to decrease the surface tension which in turn will reduce the globule size of the resulting emulsion. The accelerated physical stability test of a nanoemulsion is an important parameter that distinguishes a nanoemulsion from an emulsion that has kinetic stability and does not undergo phase separation. With the Self-Nano Emulsifying Drug Delivery System (SNEDDS) it is expected to form nanoemulsions that have high stability with no precipitation, cream formation or cracking. The test results showed that between the two formulas there was a slight precipitate which was thought to be insoluble particles from the extract. This could be due to the ethanol extract of binahong leaves containing excipients in the form of aerosols and amylum maydis. However, the F3 formula stored at room temperature remained stable for more than 14 days. Based on the results of the evaluation of the stability test of the preparation, the F3 formula was chosen as the nanoemulsion formulation formula for the ethanol extract of binahong leaves which was tested and compared its antithrombotic nd antioxidant effects with crude extracts that were not formulated.

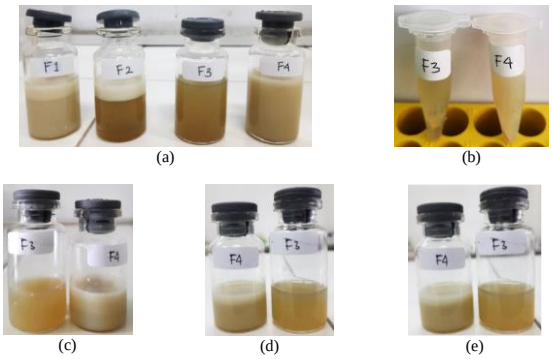


Figure 3. Results of optimalization of the formula of nanoemulsion preparation of binahong ethanol extract (BLEE). Formula 3 shows the most stable formula among the others (a). Appearance of mechanical stability test results on formulas 3 and 4 (b). The form of formula 3 and 4 nanoemulsion extract preparations after stability tests by *freeze thawing* methods is carried out for 48 hours at -4 °C (c), 25 °C (d), and 37 °C (e) temperatures, respectively.

### 3.5 Antithrombotic effect of BLEE and NBLEE

#### 3.5.1 Bleeding Time and Coagulation Time

The results of the *in vivo* test of the effect of the extract and its nanoemulsion on bleeding time and coagulation time showed that the two dosess BLEE and its nano- emulsion (NBLEE50 and NBLEE100) could significantly prolong bleeding time (Figure 4) and coagulation time (Figure 5). However, the ability to prolong the bleeding time of mice by both extracts and their nanoemulsion preparations was still lower ($p<0.01$) than the standard used (acetylsalicylic acid). Bleeding time that occurred after administration of nanoemulation preparations was longer than that of the extract, but significant bleeding time ($p<0.05$) only occurred after administration of NBLEE50 with an increase of 11.7 %. Bleeding time in BLEE50 and NBLEE50 groups were higher than large dose (BLEE100) but not statistically significant (Table 2). These data suggest increasing the dose did not prolong bleeding time.

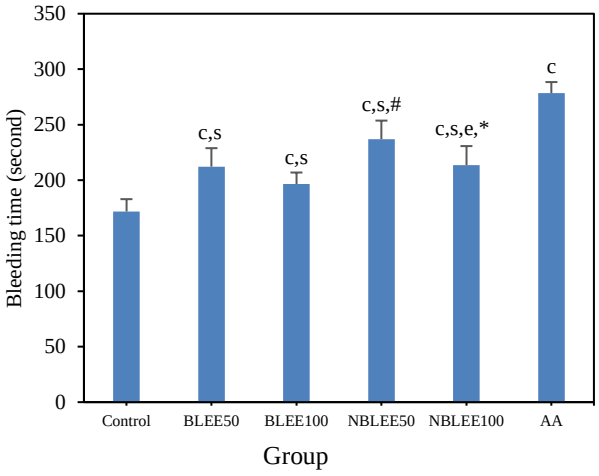


Figure 4. Bleeding time after treated wit BLEE and NBLEE c=p<0.01 compared to than that of control. *=p<0.1; #=p<0.05 compared to than that of BLEE at the same dose (BLEE50-NBLEE50 or BLEE100-NBLEE100). e=p<0.1 compared to than that of small dose (D100-D50 ; N

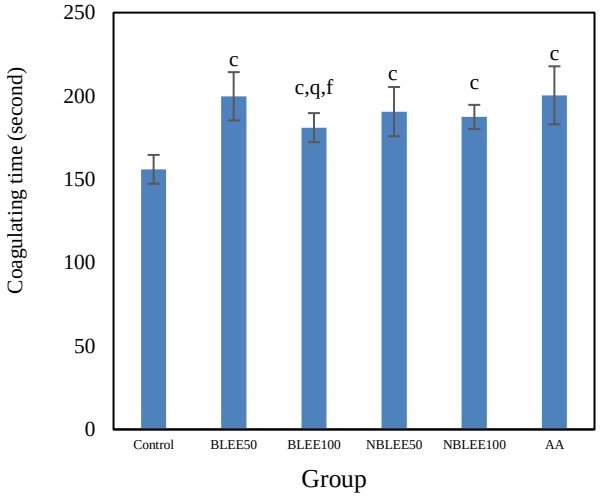


Figure 5. Coagulating time after treated with BLEE and NBLEE. c=p<0.01 compared to than that of control. f=p<0.05 compared to than that of small dose (D100-D50 ; N100-N50); AA= acetyl salicylic acid (standard).

The coagulation time of the mice given the extract and the preparation of the nanoemulsion was also lower than that of acetylsalicylic acid but not statistically significant. The blood coagulation time of mice given BLEE50 was longer than the BLEE100 and was statistically significant. Unlike what happened at the time of bleeding, increasing the dose of the BLEE did not prolong the coagulation time. As we know, the bleeding time is influenced by the size of the wound, the muscle contraction of the blood vessels, while the coagulation time is influenced by the 13 clotting factors [32]. This experimental data shows that the BLEE has activity to prolong blood clotting which is useful for preventing blood clot-related diseases.

#### 3.5.2 Blood profile

In the assay of antithrombotic activity, the platelet count is the main parameter of the antithrombotic effect. Where the active substance or drug that has activity inhibiting platelet formation will result in a decrease in the number of platelets and the risk of bleeding. On the other hand, the increase in the number of platelets and if they experience aggregation has the potential to cause platelet aggregation-related diseases such as stroke. Platelets play an important role in blood coagulation. The results of determining the blood profile of mice showed a decrease in the number of platelets in the

group that was given BLEE50 and NBLEE50 but statistically this decrease was not significant. The mean platelet volume (MPV) and the platelet distribution width (PDW) also decreased in the group given the extract and its nanoemulsion preparation and acetyl salicylic acid (Table 5). The decrease in these three blood parameters indirectly indicated the antiplatelet efficacy of BLEE.

Other blood profile data that changed was the decrease in the number of leukocytes on the administration of the extract and its nanoemulation as well as acetylsalicylic acid. Hemoglobin levels increased in the group given the nanoemulsion. Other blood parameters, i.e., the number of erythrocytes, along with other erythrocyte indices, namely MCH, MCHC, MCV, HCT, RDWa, RDW%, and LPCR were not different from the control (Table 6).

Table 5. Platelet profile after administration of BLEE50 and NBLEE50

| Group | PLT ($10^9$/L) | MPV (fL) | LPCR (%) | PDW (%) | PCT (%) |
|---|---|---|---|---|---|
| BLEE50 | 393.33±77.53 | 6.05±0.24[b] | 7.53±1.57[a] | 8.52±0.36[c] | 0.28±0.05 |
| NBLEE50 | 341.00±102.71[a] | 6.37±0.39 | 9.58±2.64 | 9.00±0.59 | 0.27±0.06 |
| AA | 311.20±56.62[c] | 6.35±0.29 | 9.60±1.33 | 8.97±0.41 | 0.22±0.05 |
| Control | 433.60±51.06 | 6.55±0.34 | 10.25±2.60 | 9.25±0.42 | 0.25±0.07 |

a=p<0.1; b=p<0.05; c=p<0.01 compared to than that of control; PLT = platelet; MPV = mean platelet volume; LPCR = Large platelet cell ratio; PDW = platelet volume distribution width, PCT = plateletcrit (Volume occupied by platelets in the blood); AA = Acetyl salicylic acid.

Table 6. Blood profile

| Group | WBC ($10^9$/L) | HGB (g/dL) | RBC ($10^{12}$/L) | MCH (pg) | MCHC (g/L) | MCV (fL) | HCT(%) | RDWa | RDW% |
|---|---|---|---|---|---|---|---|---|---|
| BLEE50 | 19.45±1.35[b] | 14.20±2.06 | 8.46±1.23 | 16.80±0.37 | 36.23±2.52 | 46.70±4.51 | 39.47±6.36 | 31.00±3.14 | 15.48±0.60 |
| NBLEE50 | 19.90±3.05[a] | 14.58±0.42[b] | 8.62±0.50 | 16.95±0.69 | 36.15±2.06 | 47.05±3.63 | 40.50±2.85 | 31.15±2.92 | 15.25±0.57 |
| AA | 18.20±1.75[a] | 14.00±0.82 | 8.60±0.68 | 16.30±0.66 | 35.72±2.54 | 45.83±2.05 | 39.43±3.74 | 30.32±1.65 | 15.58±0.66 |
| Control | 23.02±2.72 | 13.75±0.81 | 8.44±0.42 | 16.32±0.77 | 34.40±2.65 | 47.63±4.39 | 40.27±4.74 | 31.63±3.33 | 15.40±0.67 |

a=p<0.1; b=p<0.05; c=p<0.01 compared to than of control ; WBC = white blood cell; HGB = hemoglobin; RBC = red blood cell; MCH = mean corpuscular hemoglobin ; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; HCT = hematocrit, RDWa = red cell distribution width (fL); RDW = red cell distribution width (%).

### 3.5.3 Effect on platelet aggregation

Adenosine diphosphate (ADP) is a major inducer of platelet aggregation, platelet remodeling, and platelet secretion. ADP causes platelet aggregation by binding to protein receptors located on the platelet membrane. Activated platelets will secrete the contents of the granules which will increase platelet aggregation.

The platelet activity can be seen from the change in plasma absorption measured at a wavelength of 600 nm. Initial plasma uptake shows initial plasma turbidity containing unaggregated platelets. After administration of ADP, plasma absorption will decrease because the platelets in the plasma begin to form aggregates and then settle so that plasma turbidity is reduced. Drugs that have an anticoagulant effect will inhibit the decrease in plasma absorption so that the decrease is less.

Based on the test data, a decrease in plasma uptake occurred in all groups of test animals after being induced with ADP due to the formation of platelet aggregation. The smaller the decrease in plasma absorption, the higher the antithrombotic activity. Due to the small difference in the decrease in uptake, it showed a stronger inhibitory activity against ADP-induced platelet aggregation. Both doses of extract (BLEE50 and BLEE100) and its nanoemulsion (NBLEE50 and NBLEE100) could significantly inhibit platelet aggregation ($p<0.05$) with each inhibition of 72-76% (Figure 6). The inhibitory power of the two doses was not different compared to the nanoemulsion preparations. These data indicate that BLEE formulated in the form of nanoemulsions in this study has not succeeded in increasing the anti-

platelet aggregation effect. The inhibition of platelet aggregation of the two doses and their naoemulsion preparations did not differ compared to the standard.

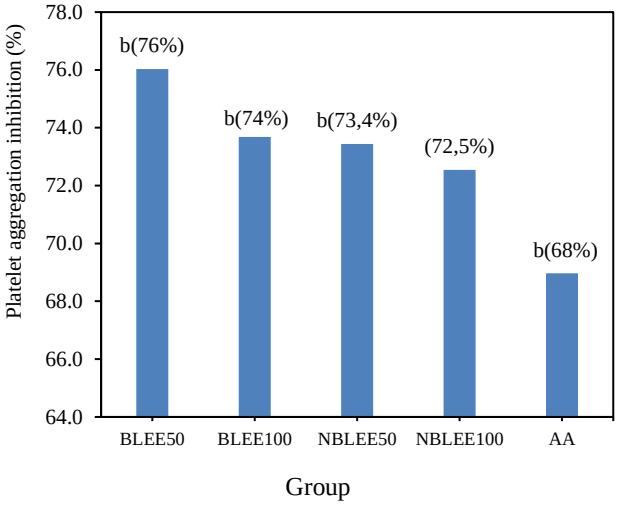


Figure 6. Platelet aggregation inhibition. b=p<0.05 compared to than that of control.

### 3.5.4 Effect on lipid peroxidation

Lipid peroxidation inhibition test aims to determine the antioxidant effect of BLEE. When lipid peroxidation occurs from the cell membrane, malondialdehyde (MDA) is formed. MDA can be produced by enzymatic and non-enzymatic process and it is a useful marker to determine lipid peroxidation process [33]. The MDA test can be used to measure peroxidation that occurs in lipid membranes. The MDA profile in serum serves as a marker of cellular damage caused by free radicals. The higher the levels of free radicals, the higher the levels of MDA formed. The higher the MDA level, the less efficacious the test compound was. In this study, lipid peroxidation was determined in plasma, liver and brain. The results showed that both BLEE and NBLEE inhibited lipid peroxidation very significantly (p<0.01) with an inhibitory power ranging from 24-25% in plasma and liver samples. The lipid peroxidation inhibitory power of both doses of the test extract and its nanoemulsion preparation (about 25%) was lower and significantly (p<0.01) compared to the standard used in this study, namely ascorbic acid (46%). In the test with brain specimens, significant lipid peroxidation inhibition (p<0.05) occurred after administration of high-dose of extract (BLEE100) and nanoemulsion preparations (NBLEE50 and NBLEE100). The inhibition of lipid peroxidation in the brain by the test extract and its nanoemulsion preparation was also lower (p<0.01) compared to the standard. These data indicate the ethanol extract of binahong leaves has efficacy as an antioxidant. This data is in line with the results of the DPPH test which shows the BLEE is classified as a strong antioxidant. The formulation of the test extract in the form of nanoemulsion in this study has not succeeded in increasing the inhibitory effect of lipid peroxidation.

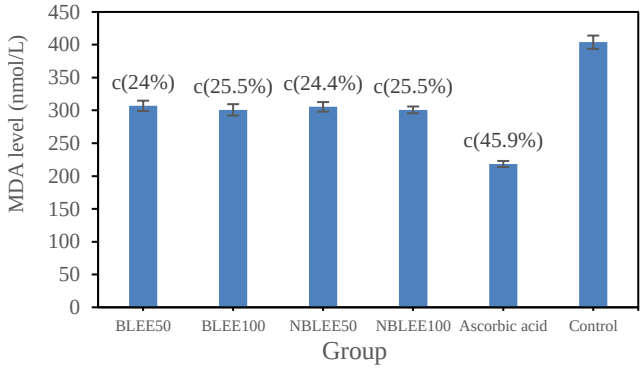


Figure 7. MDA level in plasma. c=p<0.01 compared to than that of control

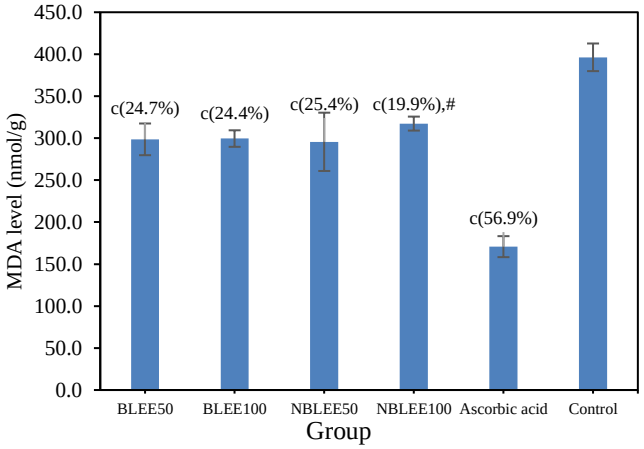


Figure 8. MDA level in liver. c=p<0.01 compared to than that of control. #=p<0.05 compared to than that of BLEE at the same dose (BLEE50-NBLEE50 or BLEE100-NBLEE100)

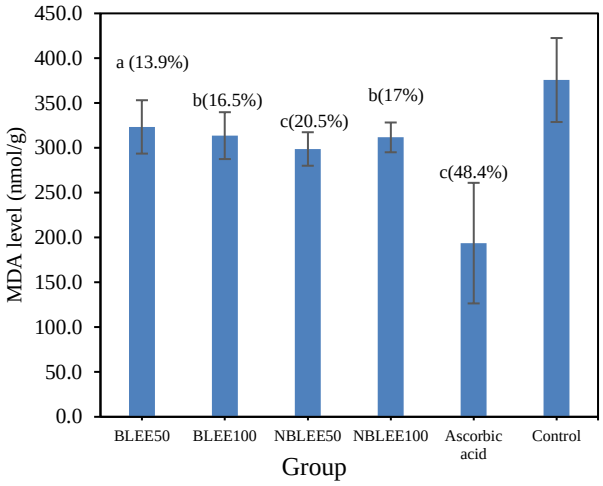


Figure 9. MDA level in brain. a=p<0.1; b=p<0.05; c=p<0.01 compared to than taht of control

The presence of free radicals in the body has the potential to cause tissue damage and cause various degenerative diseases. Normally in the body there are endogenous antioxidants such as catalase, superoxide dismutase (SOD) and glutathione peroxidase. The reduced activity of these enzymes can lead to lipid peroxidation which results in the stimulation of platelet aggregation. Platelet aggregates lead to the formation of blood clots which can threaten the emergence of related diseases such as stroke.

### 3.5.5 Nitric oxide levels

Nitric oxide (NO) is a radical that plays an important role in tissue damage. NO is synthesized endogenously and is found in many cells, including vascular endothelial cells, smooth muscle cells, platelets, nerve cells, and in phagocytic cells [34], [35]. This nitrogen monoxide compound is able to stimulate and inhibit lipid oxidation. This compound also has a dual role, either as a pro-oxidant or as an antioxidant which is very dependent on the oxidative status in the tissue [36]. Its effect on the oxidation of membrane lipids and lipoproteins can increase the risk of plaque formation in the endothelial lining - the inner walls of blood vessels. Plaque formation is usually associated with vascular related diseases such as stroke. Oxidation of membrane lipids has the potential to form eicosanoids which have an impact on the development of vascular inflammatory diseases.

The test results showed that both the extract dose and the nanoemulsion preparation could significantly inhibit NO formation ($p<0.01$). The inhibitory power of the two doses of extract and nanoemulsion preparation (23.77–28.29%) was not different from the standard used, namely ascorbic acid (26.93%). The inhibition of NO formation increased in the nano emulsion preparation containing the extract at dose of 50 mg/kg bw (28.29% vs 26.52%), but decreased in the nanoemulsion preparation containing the extract at dose of 100 mg/kg bw (27.67% vs. 23,77%). These data indicate that the formulation of extracts in the form of nanoemulsions can affect the efficacy of an extract. In addition, these data add to the antioxidant properties of the ethanol extract of binahong leaves.

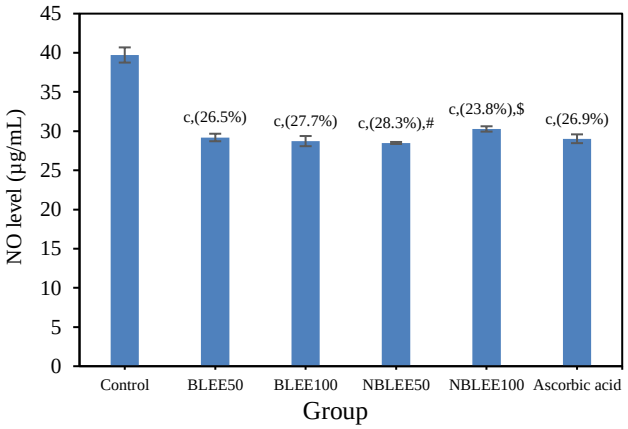


Figure 10. Nitric oxide level. c=p<0.01 compared to than of control. #=p<0.05; $=p<0.01 compared to than of BLEE at the same dose (BLEE50-NBLEE50 or BLEE100-NBLEE100)

## 4 Conclusions

Based on the results of this study, it can be concluded that the ethanol extract of binahong leaves at a dose of 50 mg/kg body weight has antithrombotic properties based on the parameters of bleeding time, coagulation time, Mean Platelet Volume, Platelet Distribution Width and inhibition of platelet aggregation. The formulation of the ethanol extract of binahong leaves in the form of nanoemulsions is

also antithrombotic but has not been able to increase the antithrombotic effect. The ethanol extract of binahong leaves is efficacious as an antioxidant with a strong category in the DPPH test. In vivo ethanol extract at doses of 50 and 100 mg/kg bw and its nanoemulsion preparations had antioxidant properties in the lipid peroxidation inhibition test and significantly inhibited the formation of NO. Henceforth, the ethanolic extract of binahong leaves can be made as herbal medicine to treat diseases related to blood clots.

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## 6 Conflicts of Interest

The author has no funding or any other conflict of interest in this research.

## 7 Funding

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## 8 Ethical Approvals

Before the test trial was carried out, first all tests to the test animal model were evaluated and given permission by the Ethics Committee for the Use of Test Animals of the Bandung Institute of Technology (ITB) with a Certificate Number: 04/KEPHP-ITB/11-2021.

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