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Potential of Binahong Leaf Extract (*Anredera cordifolia*) for Anemia Treatment in Anemic Rat Model

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Abstract

Anemia, characterized by low hemoglobin (Hb) and erythrocyte counts, can be induced by reactive oxygen species (ROS) or known as hemolytic anemia. Binahong (Anredera cordifolia) has been explored for its potential in managing anemia; yet, its efficacy against ROS-induced anemia remains under investigated. This study, conducted from January to May 2023, aimed to evaluate the potential of Binahong leaf extract (BLE) in treating anemia caused by oxidative stress. Anemia was induced by administering NaNO2 to 24 rats (Rattus norvegicus), followed by the administration of BLE at doses of 50,100, and 200 mg/kgBW for seven days. Hemoglobin levels were measured using Sahli method and erythrocytes count were determined using a Hemocytometer at three stages: pre anemia (HA), before-treatment (H0), and after-treatment (H7). Results showed that BLE significantly increased the Hb level by 1.3g/dL (p=0.000), 3.87g/dL (p=0.034), and 4.53g/dL (p=0.016) at 50 mg/kgBW, 100mg/kgBW, and 200 mg/kgBW, respectively, after treatment. Additionally, a dose of 200 mg/kgBW significally increased the erythrocyte count by 3.84 x 106 L/mm3 (p=0.033). These findings suggested that BLE has the potential to improve Hb levels and erythrocyte counts in ROS-induced anemia, indicating a promising natural approach to managing anemia.

Keywords: *Anredera cordifolia*, erythrocyte, hemoglobin, *Rattus novergicus*

Introduction

The World Health Organization (WHO) estimates that anemia afflicts 2 billion individuals worldwide and is predominantly caused by iron deficiency.[1] In Indonesia, the prevalence of anemia is alarmingly high, with approximately 42% of children under the age of 5 years, 40% of pregnant women, and 30–32% of adolescents experiencing anemia.[1,2]

Anemia presents a significant public health challenge, characterized by reduced hemoglobin, hematocrit levels, and/or erythrocyte count.[3] Changes in these parameters can induce hypoxia and increased oxidative stress, marked by elevated levels of reactive oxygen species (ROS).[3] Iron deficiency has been traditionally linked to anemia cases, but it is essential to acknowledge that oxidative stress can also contribute to its development. Erythrocytes are particularly vulnerable to oxidative stress due to their primary role in oxygen transportation.[4] Within the bloodstream, erythrocytes are constantly exposed to ROS, such as hydrogen peroxide and superoxide, leading to hemolytic anemia.[5] The ROS can oxidize hemoglobin, produce methemoglobin, and cause cell damage through lipid peroxidation, ultimately leading to hemolysis and reduced hemoglobin levels.[5] Currently, anemia is primarily treated with iron-based drugs, which may cause nausea, vomiting, diarrhea, and constipation. Furthermore, the

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primary treatment for hemolytic anemia is blood transfusions, where repeated transfusions can lead to iron accumulation within the body.[5]

The Madeira vine, or *Anredera cordifolia* (*Tenore*) *Steenis*, is a succulent climbing vine member of the Basellaceae family. The Madeira vine, known as Binahong in Indonesia, is a well-known traditional medicinal herb valued for healing wounds and curing various ailments.[6] These plants contain many flavonoids in their leaves, stems, tubers, and flowers, which are potent antioxidants and promote erythropoiesis and immunostimulation.[7] The flavonoids also play a pivotal role in managing anemia by preserving heme, which contains iron ions (Fe^{2+}) crucial for producing hemoglobin.[8] Furthermore, ascorbic acid in Binahong leaves accelerates iron absorption up to four times faster, particularly in acidic conditions.[9,10]

Binahong has long been utilized as a traditional medicine in Indonesia. It has been extensively researched, focusing on its antioxidant and antibacterial characteristics and its use to treat bleeding.[11-13] However, there is insufficient evidence of its efficacy in treating ROS-induced anemia.

Therefore, this research aims to investigate whether Binahong leaf extract can improve anemia, especially oxidative stress anemia, by increasing Hb and erythrocyte counts in anemic models. These results could guide further research into developing Binahong leaf extract as a new treatment for anemia.

Methods

The study received approval from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Universitas Kristen Krida Wacana (UKRIDA), with the approval numbers 1308/SLKE-IM/UKKW/FKIK/KE/VIII/2022 and 1385/SLKE-IM/UKKW/FKIK/KE/X/2022. The experiment used male rats (*Rattus norvegicus*) sourced from IPB. They were in verified good health and consistent morphology, weighing between two and three months. The experimental study was conducted from January to June 2023 in the UKRIDA experimental animal laboratory.

After a seven-day adaptation period, anemia was induced in the rats by oral administration of $NaNO_2$ at 25 mg/200g BW per day for 14 days using a gastric probe.[14] Following the adaptation period, the rats were divided into four groups: a negative control group (K1), a treatment group (P1), a treatment group (P2), and a treatment group (P3). All groups received standard feed daily. The negative control group received distilled water, and the treatment group received ethanol extract of Binahong leaves with 50 mg/kgBW (P1), 100 mg/kgBW (P2), and 200 mg/kgBW (P3). The sample size was determined using the Federer formula: (t-1) (n-1) ≥15, where t is the number of treatment group, and n is the number of samples in each treatment groups. A total of 24 rats were used in this study. Furthermore, blood (1–2 mL) was collected from the rat's retro-orbital sinus using a sterile capillary pipette. The collected blood was then transferred to an EDTA-containing microtube to facilitate subsequent quantification of hemoglobin levels and red blood cell counts.

Binahong extract was prepared by cleaning and air-drying the leaves over five days. Subsequently, the dried leaves were finely powdered using a blender. In a maceration jar, 40 grams of powdered Binahong leaves underwent extraction over two 24-hour intervals using 1000 milliliters of 70% ethanol. The resultant mixture was then filtered using filter paper. The filtrate was evaporated using a rotary evaporator at 220 mmHg pressure and 60°C. Before its use in the experiment, the extract was stored in a sealed, clean container in a refrigerator at 4°C.[15]

The chemical reagents used in this study included 0.1 N hydrochloric acid (HCl) for organic compound hydrolysis, glacial acetic acid (CH_3COOH) for bacterial growth inhibition, sulfuric acid (H_2SO_4) for organic compound oxidation, Mayer's reagent for alkaloid detection, Hayem's solution for hemoglobin level assessment, and ethanol as a solvent.[16,17]

Hemoglobin (Hb) concentration was determined using the Sahli method. Briefly, 2 mL of 0.1 N HCl solution is added to a Sahli tube up to the two-mark. Twenty microliters (µL) of blood are then aspirated into a Sahli pipette up to the 20 µL mark and transferred to the tube containing the HCl solution. Following a 5–10-minute incubation for hematin formation, distilled water is added dropwise until the sample color matches a standardized Hb solution within the Sahli tube. The Hb level was then read from the meniscus of the liquid on the g% scale of the Sahli tube, representing the Hb concentration in grams per 100 mL of blood.[18]

Erythrocyte counts were determined using an improved Neubauer hemocytometer and a red blood cell pipette. Blood was drawn to the 0.5 mark in the pipette, diluted with Hayem's solution to the 101 marks, and mixed vigorously for 3–5 minutes. After discarding the first

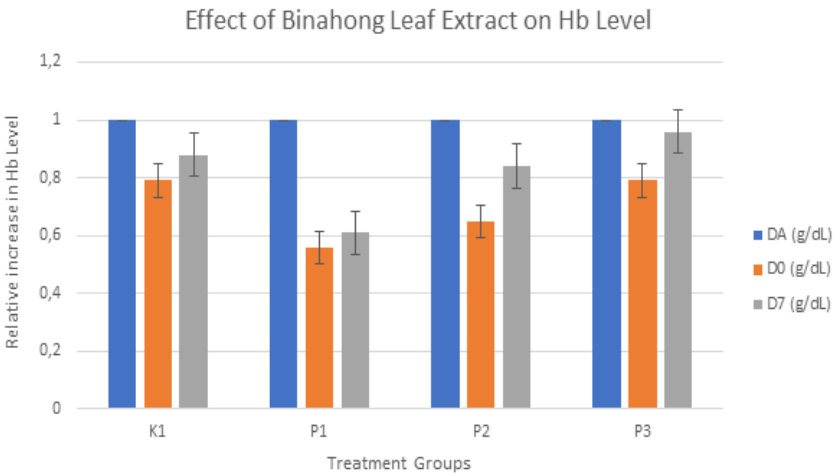

Figure 1 Relative Increase in Hemoglobin (Hb) Levels Following Binahong Leaf Extract (BLE) Treatment

Groups: negative control (K1, distilled water), and treatment groups receiving BLE at 50 mg/kgBW (P1), 100 mg/kgBW (P2), and 200 mg/kgBW (P3)

two drops, a blood droplet was placed on the hemocytometer and covered with a coverslip. Erythrocytes were counted under a microscope at 400x magnification within five large squares, and the total count was calculated considering the dilution factor and volume of the large squares.[18]

Statistical analysis was conducted to examine the average erythrocyte count and Hb levels at different time points before the sodium nitrite administration (DA), 14 days after the administration (D0), and seven days after the treatment (D7). The data was initially assessed for normality and homogeneity using the Shapiro-Wilk test. A normal distribution was considered if the p-value was >0.05, and homogeneity was considered if the p-value was >0.05. A paired t-test was used to evaluate the dependent data. If the p-value was <0.05, H1 was accepted, and H0 was rejected, indicating a significant difference between the analyzed data groups.[19,20]

Results

The ethanol extraction process from 40 grams of Binahong leaf powder yielded 150 milliliters of extract. To determine the yield, 1 milliliter of extract was oven-dried, resulting in a residue weight of 0.03 grams. This corresponds to an extract yield of 11.25%. Figures 1 and 2 present the mean hemoglobin (Hb) levels and red blood cell (erythrocyte) counts across the four groups at three time points: before sodium nitrite administration (DA), after 14 days of administration (D0), and after seven days of treatment (D7).

The mean differences in hemoglobin (Hb) and red blood cell (erythrocyte) counts for each group are presented in Tables 1 and 2, comparing (1) DA to D0, (2) D0 to D7, and (3) DA to D7.

Table 1 shows the difference in mean Hb levels between the D0 and D7 groups, with the highest value observed in the P3 anemic rat

Table 1 Data of the Difference Mean Hb Level between HA, H0, and H14*

Treatment Groups	ΔHA(g/dL)	ΔH0 (g/dL)	ΔH14
K1	3.00	1.20	1.80
P1	4.80	1.35	3.45
P2	3.87	3.87	0.00
P3	2.60	4.53	-1.93

*: A negative control group (K1) and a treatment group received an ethanol extract of Binahong leaves with 50 mg/kgBW (P1), 100 mg/kgBW (P2), and 200 mg/kgBW (P3)

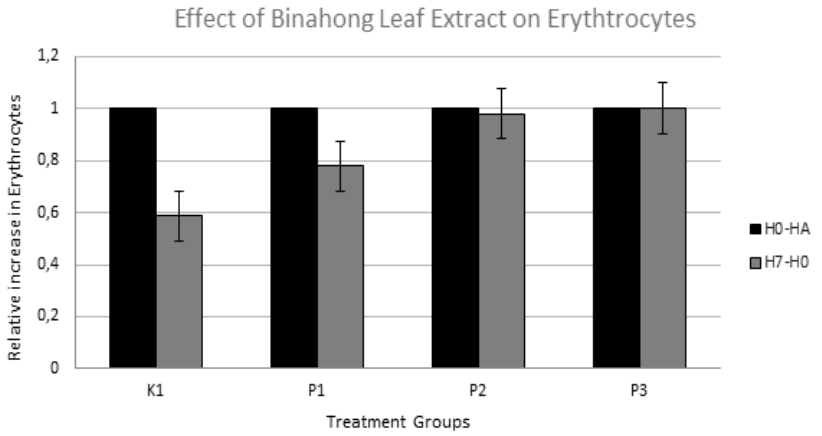

Figure 2 The relative increase in erythrocyte counts after BLE administration compared to the decrease in erythrocyte count in anemia. A negative control group treated using distilled water (K1), a group treated with ethanol extract of Binahong leaves with 50 mg/kg BW (P1), 100 mg/kg BW (P2), and 200 mg/kg BW (P3)

group at 4.53 g/dL. This indicates the capacity of BLE to recover Hb levels to their pre-anemic state and surpass the Hb level of the control group, suggesting optimal improvement of anemia conditions.

Table 2 shows the mean red blood cell count difference between the H0 and H7 groups, with the highest value observed in the P3 anemic rat group at 3.84 x 10^6 L/mm^3 of blood.

The Shapiro-Wilk statistic was used to test the normality of the mean Hb levels and red blood cell count data due to the available data being less than 30. The statistical test revealed that mean Hb levels and red blood cell counts at DA, D0, and D7 were normally distributed, with p-values greater than 0.05. Furthermore, according to Levene's test statistic, the data showed homogeneous variance for all groups (DA, D0, and D7) with a p-value greater than 0.05.

A paired t-test was used to see if there were any differences in the mean Hb and erythrocyte counts for each group between D0 and D7. The paired t-test results for the mean Hb levels between D0 and D7 in the P1, P2, and P3 groups had a p-value less than 0.05, indicating that the administration of ethanol extracts of Binahong leaves can increase the Hb levels in white rats. However, the paired t-test results for the mean Hb levels between D0 and D7 in the K1 groups had a p-value greater than 0.05, indicating no significant variations in mean Hb levels between D0 and D7, as shown in Table 3.

The paired t-test results for mean erythrocyte counts between D0 and D7 in each group had a p-value greater than 0.05, indicating no significant differences between D0 and D7 for all groups except the P3 group. The paired t-test results of the P3 group had a p-value less than 0.05, indicating a significant difference in mean erythrocyte counts between D0 and D7, as shown in Table 4.

Table 2 Data of the Mean Erythrocyte Count Difference between HA, H0, and H7*

Treatment Groups	ΔHA (µLx10^6)	ΔH0 (µLx10^6)	ΔH14 (µLx10^6)
K1	3.87	2.27	1.60
P1	2.94	2.29	0.65
P2	3.03	2.97	0.06
P3	3.84	3.84	0.000

*: A negative control group (K1) and a treatment group received an ethanol extract of Binahong leaves with 50 mg/kg (P1), 100 mg/kgBW (P2), and 200 mg/kgBW (P3)

Table 3 Statistical Analysis Results of Mean Hb Level*

Effect of Treatment	Mean±SD (g/dL)	p-value
K1		
Day-0	11.40±0.14	0.495
Day-7	12.60±0.85	
P1		
Day-0	14.65±1.59	0.000**
Day-7	16.00±1.58	
P2		
Day-0	13.33±1.90	0.034**
Day-7	17.20±1.22	
P3		
Day-0	12.47±1.30	0.016**
Day-7	17.00±1.22	

*: A negative control group (K1), a treatment group, received ethanol extract of Binahong leaves with 50 mg/kgBW (P1), 100 mg/kgBW (P2), and 200 mg/kgBW (P3). **: SD = standard deviation; p<0.05 with paired t-test

Discussion

Anredera cordifolia leaf powder was extracted using 70% alcohol to extract chemical components such as flavonoids, ascorbic acid (Vitamin C), and other substances.[18] The extraction process produced 150 mL of extract from 40g of *Anredera cordifolia* leaf powder, with a yield of 11.25%, which means the ratio of the final product's dry weight to the raw material's weight. A higher yield indicates a higher concentration of extracted chemicals from the raw material.

Nitrate (NO_3^-) and nitrite (NO_2^-) are inorganics ions naturally present in the nitrogen cycle. Exposure to nitrate or nitrite can occur through various sources, such as well water, food, workplace environment occupational exposure, and certain medications. In this study, the rats were given sodium nitrite ($NaNO_2$) for 14 consecutive days at a dose of 25 mg/200g body weight to induce anemia, characterized by reduced hemoglobin levels and a drop in red blood cell count across all treatment groups. Sodium nitrite is used to produce nitrite ions (NO_2^-) and increases the oxidation of the ferrous iron (Fe^{2+}) in deoxyhemoglobin to the ferric (Fe^{3+}) producing methemoglobin and triggers oxidative stress and cellular damage, including red blood cell damage, which further contributes

Table 4 Statistical Test Results of Mean Erythrocyte Counts Data

Effect of Treatment	Mean ± SD ($\mu L \times 10^6$)	p-value
K1		
Day-0	4.31±0.76	0.095
Day-7	6.58±1.40	
P1		
Day-0	5,05±0.65	0.235
Day-7	7.34±1.57	
P2		
Day-0	5.63±1.23	0.109
Day-7	8.61±1.31	
P3		
Day-0	5.65±1.26	0.033**
Day-7	9.49±1.05	

*: A negative control group (K1), a treatment group, received ethanol extract of Binahong leaves with 50 mg/kg BW (P1), 100 mg/kg BW (P2), and 200 mg/kg BW (P3). **: SD = standard deviation; p<0.05 with paired t-test

to the development of anemia.[14,22] Under normal circumstances, only small amount of iron oxidizes to the ferric (Fe^{3+}) state during the routine delivery of oxygen to tissue and the maintenance of methemoglobin levels is primarily facilitated by cytochrome b5 reductase (CYB5R) and the NADH-dependent methemoglobin reductase system.[23]

The CYB5R enzyme is involved in reducing methemoglobin by catalyzing the reduction of methemoglobin (Fe^{3+}) back to functional hemoglobin (Fe^{2+}), which is important for oxygen transport in red blood cells.[20] However, exposure to nitrite ions, which caused an increase in ROS in this study, decrease CYB5R activity through ROS interaction with the heme-binding domain of CYB5R.[20]

Previous research shows that Binahong leaf extract is rich in antioxidants such as flavonoids, ascorbic acid (Vitamin C), and other compounds.[18] The presence of these large amounts of antioxidants is expected to restore the oxidant-antioxidant balance and prevent oxidative stress, optimizing the function of CYB5R in maintaining blood levels methemoglobin in the body and provides a conducive environment for the formation of healthy red blood cells, which in turn improves the previous anemia condition.

Based on the results of this study, *Anredera cordifolia* leaf extract is appears to be effective in treating anemia in P3 group mice, showing a significant increase in Hb levels and erythrocyte counts, particularly at a dose of 200 mg/kgBW. It is suggested that antioxidants such as flavonoids, ascorbic acids, and other compounds in *Anredera cordifolia* leaf extract are believed to improve anemia conditions caused by ROS.

However, the underlying mechanisms have not been explored, and further research is needed to precisely understand the processes that underlie the improvement of anemia with *Anredera cordifolia* leaf extract administration.

In conclusion, Anredera cordifolia leaf extract, particularly at a dose of 200 mg/kgBW, effectively improves hemoglobin levels and erythrocyte counts in rats with sodium nitrite-induced anemia.

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