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INVESTIGATION OF TOTAL PHENOLIC CONTENT, FLAVONOID CONTENT, AND HEMOSTATIC ACTIVITY OF BEETROOT (*Beta vulgaris*. L) EXTRACT IN HEPARIN-INDUCED THROMBOCYTOPENIA RAT

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ABSTRACT

The goal of the research was to find out the concentration of phenolic and flavonoid content and also to identify the hemostatic activity of the ethanolic extract of beetroot (*Beta vulgaris* L) in heparin-induced thrombocytopenia rats. The identification of total phenol content using Folin-Ciocalteu as reagent and flavonoid content were determined by spectrophotometer and continued to the determination of hemostatic activity using heparin as an inducer and then determined the bleeding time, platelet count, and prothrombin time. The results showed that the ethanol extract of beetroot had a total phenolic content of 312.2 mg GAE/g extract and a flavonoid content equivalent to 38.5 mg quercetin equivalent/g. The investigation of bleeding time at doses of 100 mg/kg BW, 200 mg/kg BW, and 400 mg/kg BW was significantly different ($p<0.05$) compared to the negative group. The dose of 400 mg/kg BW reduced the bleeding time by 5.90±0.11 minutes, which was substantially different from the other doses ($p<0.05$). At a dose of 400mg/kg BW, the greatest platelet count number was seen, which was statistically different from the control group ($p<0.05$). The prothrombin Time (PT) values at a dose of 400 mg/kg BW likewise revealed increased activity and were significantly different ($p<0.05$) from the negative group. The ethanol extract of beetroot (Beta vulgaris L) possesses hemostatic action and is dose-dependent, with a total phenolic concentration of 312.2 mg GAE/g extract and a flavonoid content equivalent to 38.5 mg quercetin equivalent/g extract.

Keywords: Beetroot (*Beta vulgaris* L.), Hemostatic, Bleeding Time, Platelet, Prothrombin time.

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INTRODUCTION

Hemostasis is the intricate cycle of blood clump development and addresses a planned reaction to vessel injury.[1] This is refined by the organized endeavors of three related systems: the platelet, the vascular system, and the coagulation periods of hemostasis.[2] Bleeding disorders are caused by a breakdown in hemostasis, resulting in an increased risk of bleeding. Platelet abnormalities, coagulation deficiencies, or a combination of the two are the most common causes.[3] Since ancient times, Indonesia has had several plants that can be used as sources of therapeutic compounds.[4] Phytochemical compounds that are found in beetroot are tannin saponins, alkaloids, flavonoids, terpenoids, and steroids. Several minerals are also present in beetroots, such as magnesium (Mg), Iron (Fe), copper (Cu), potassium (K), sodium (Na), manganese (Mn), zinc (Zn), and calcium (Ca).[5] Calcium has been shown to speed up the production of thrombin and increase the production of fibrin.[6] It indicated that beetroot has a potential effect coagulation cascade. Previous research has revealed that beetroot methanol extract improves the production and growth of blood cells.[7] Based on the mentioned data, it is necessary to determine the hemostatic effects of ethanol extracts of beetroot by using a heparin-induced thrombocytopenia rat as an animal model and then measuring the bleeding time, platelet count and prothrombin time (PT) as well as the dried samples characterization, and the concentration of total phenol and flavonoid were also examined in this study.

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EXPERIMENTAL

Materials

Beetroot Tuber, Heparin Sodium (Fahrenheit), Folin-Ciocalteu reagent (Sigma), sodium Carbonate (Merck), aluminium chloride/AlCl3 (Merck), distilled water, quercetin (Merck), methanol (Merck), and gallic acid (Merck).

Plant Collection and Extraction of Beetroot

The beetroot tuber was collected at the local market in Padang Bulan, Sumatera Utara, Indonesia. The plant samples were verified by the Indonesian Institute of Science's Research Centre of Biology in Bogor, Indonesia with the Certificate Number: 294/IPH.1.01/If.07/V/2017. A total of 300 g of dried beetroot was crushed, and ethanol solvent 96 percent was passed through a sample drop by drop for 10 days in a percolator. The results were evaporated in a rotary evaporator at 50°C, then dried in a water bath.[8]

Dried Samples Characterization

The procedure of dried samples characterization according to the guidelines released by The National of Drug and Food Control, Indonesia.[9]

Qualitative Phytochemical Identification

The qualitative phytochemical identification is conducted to find out the chemical constituent in beetroot extract such as flavonoids, alkaloids, saponins, tannins, glycosides, and steroids/terpenoid.[10,11,12]

Calculation of Total Phenol Concentration

The folin reagent was used to assess the sample's total phenol concentration (TPC). A 100 µL beetroot extract (500 µg/ml) were mixed with 7.9 mL distilled water and 0.5 mL folin-reagent ciocaleu's (1:10 v/v) and vortex-mixed for 1 minute. After mixing, 1.5 mL of 20% aqueous sodium bicarbonate was added, and the mixture was allowed to sit for 90 minutes while being shaken intermittently. The absorbance was measured at 775 nm using a spectrophotometer. Total phenolic content is calculated using gallic acid equivalent in milligrams per gram of extract. A blank solution of methanol was used. Triplicates of each experiment were performed.[13]

Calculation of Total Flavonoid Concentration

The total flavonoid concentration was determined using a spectrophotometer and the colorimetry method. 25 mg of beetroot ethanol extract was dissolved in 25 ml of methanol, then diluted to 300 ppm. After creating 2 ml of the sample at a concentration of 300 ppm, the solution was mixed with 0.1 ml of AlCl3, 0.1 ml of sodium acetate, and 2.8 ml of purified water. A visible spectrophotometer was used to measure the absorbance at a wavelength of 750 nm. Milligrams of equivalent quercetin per gram of material (mg Q/g) were used to calculate the flavonoid content.[14]

Animals and Blood Collection

The animal model used in this study was 25 male Wistar rats, weighing 180-220 g. The blood sample was collected from the tail for bleeding time test and from vein cava inferior for hematological properties.

Treatment Regime

The rats were put into five groups at random, each group consisting of five rats. Rats were induced with heparin at a dose of 270 IU / 200 g BW / day for 10 days to induce thrombocytopenia condition then continue to give beetroot extract for 10 days[15]. On the final day of the experiment, the rats in groups 1 to 5 measured the bleeding time by Duke Method, platelet count using Sysmex Automated Hematology Analyzer XN 450, and prothrombin time using Coatron® A4 Fully automated Hemostasis Analyzer.[16,17,18] The test groups are divided into the following distribution:

1. Group 1 was a normal control group without treatment
2. Group 2 was a negative control group induced with heparin at a dose of 270 IU / 200 g BW/day for 10 days, then given CMC Na 0.5% BW for 10 days
3. groups 3, 4, and 5 were the treatment groups induced with heparin at a dose of 270 IU/200g BW/day for 10 days, then given 2% beetroot extract with various dosages, namely 100 mg /kg, 200 mg/kg, and 400 mg/kg BW per day for 10 days

Statistical Analysis

The data were analyzed using the statistical method of ANOVA with Tukey's Multiple Comparison Test. *P* values significant value were set at 0.05. The data expressed as the mean ± SD.

RESULTS AND DISCUSSION

Dried Samples Characterization

Table-1: The Results of the Beetroot Dried Samples Characterization Examination

No.	Examination	Results (%)
1.	Water content	8.3
2.	Water-soluble content	31.62
3.	Ethanol soluble content	33.83
4.	Total ash content	6.38
5.	Acid insoluble ash content	0.79

Qualitative Phytochemical Identification Result

Phytochemical screening results showed that booth dried samples and ethanol extract beetroot positively contains flavonoids, alkaloids, saponins, tannins, glycosides, and steroids/terpenoid.

Table-2: Phytochemical Identification Result

No.	Content	Ethanol Extract
1.	Flavonoids	+
2	Tannins	+
3.	Saponins	+
4.	Steroids/terpenoid	+
5.	Glycosides	+
6.	Alkaloids	+

Determination of Total Phenolic and Flavonoid Contents

Most of the phytochemical constituents found in plants are phenolic compounds. Spectrophotometry using Folin-Ciocalteu reagents can be used to determine the total phenolic content of the extracts. Gallic acid was used as a comparison phenolic compound. The equation of line produced based on the results was: y=0, 00150 X + 0, 0221; r2 = 0.998. The ethanol extract of beetroot extract has a total phenolic content of 312.2 mg GAE/g extract. The quercetin calibration curve was calculated using the equation y = 0.02246 x + 0.12217; R^2 = 0.999.The results of total phenolic and flavonoid content can be seen in table 3.

Table-3: Results of Total Phenol and Flavonoid Content

Parameters	Results
Total Phenolic Contents	312.2 mg GAE/g extract.
Total Flavonoid Content	38.5 mg quercetin equivalent/g extract

Determination of Hemostatic Effects of Ethanol Extracts of Beetroot Results

Table-4: Results of In Vivo Bleeding Time of Rats (mean ± SD, n=5)

No	groups	Bleeding Time (minute)					Mean ± SD
1	Normal Group	4.6	4.75	4.58	4.56	4.7	4.63 ± 0.08$^{\#}$
2	Negative Control Group	15.43	15.46	14.96	15.06	15.25	15.23±0.22*
4	Beetroot extract 100 mg/kg BW	10.16	10.35	10.25	10.15	10.1	10.20±0.09*$^{\#}$
5	Beetroot extract 200 mg/kg BW	7.15	7.2	7.31	7.08	7.33	7.21±0.10*$^{\#}$
6	Beetroot extract 400 mg/kg BW	6.08	5.85	5.95	5.76	5.9	5.90±0.11*$^{\#}$

(*): significantly different (P <0.05) with normal control. (#): significantly different (P <0.05) against negative control

Table-5: The Results of the Calculation in the Rat Platelet Count (mean ± SD, n = 5)

No	Groups	Platelet count (x10^3 /µl)					Mean ± SD
1	Normal Group	698	701	693	684	713	697.8±10.66$^{\#}$
2	Negative Control Group	143	138	131	136	145	138.6±5.59*
4	Beetroot extract 100 mg/kg BW	165	156	172	158	159	162±6.51*$^{\#}$
5	Beetroot extract 200 mg/kg BW	458	478	467	482	492	475.4±13.22*$^{\#}$

6	Beetroot extract 400 mg/kg BW	621	645	619	638	633	631.2±11.09*#

(*): significantly different (P <0.05) with normal control. (#): significantly different (P <0.05) against negative control

Table-6: Result of Calculation of PT (Prothrombin time) (mean + SD, n = 5)

No	Groups	*Prothrombin time* (seconds)					Mean ± SD
1	Normal Group	21.2	19.5	18.4	20.5	21.5	20.22±1.2#
2	Negative Control Group	45.5	39.2	43.6	42.5	43.4	42.84±2.3*
4	Beetroot extract 100 mg/kg BW	31	30.3	31.6	30.4	32.9	31.24±1.06*#
5	Beetroot extract 200 mg/kg BW	26.7	26	25.6	25	27.4	26.14±0.9*#
6	Beetroot extract 400 mg/kg BW	23.8	25	24.7	24.9	25	24.68±0.5*#

(*): significantly different (P <0.05) with normal control. (#): significantly different (P <0.05) against negative control

Heparin-induced thrombocytopenia (HIT) is a condition that is caused by the formation of antibodies against a specific protein complex against platelets, platelet factor 4 (pf4) -heparin complex resulting in a decrease in the number of platelets.[19] Thrombocytopenia can increase bleeding time, a low platelet count causes the normal hemostasis process to be disrupted resulting in an increase in bleeding time.[20] The results of statistical testing of the bleeding time of rats at several doses of beetroot extract indicated that the dose of 100 mg/kg, 200 mg/kg, and 400 mg/kg of body weight differed significantly ($p<0.05$) against the negative control group. The result indicates that beetroot extract can decrease the bleeding time of heparin-induced rats. Okoro et al in the year 2017 reported that the beetroot tuber contains some minerals such as calcium.[5] The calcium can accelerate the formation of thrombin and will stimulate the formation of fibrin threads, so that blood can clot and reduce bleeding time.[21] Calcium is one of the blood coagulation factors.[22] In the coagulation pathway, calcium ions play a role in the conversion process of factor x to factor Xa and also the change of prothrombin to thrombin.[23] Based on the research of Nakashima, et al, in the year 2006, rats with hypocalcemia experienced prolonged bleeding.[24] This suggests that calcium has an important role in the hemostasis process. The content of tannin can reduce bleeding time, tannins have been proven in accelerating tissue contraction and damaged capillaries with their astringent effect and can accelerate blood clotting.[25] According to dandjesso in the year 2012, tannins are a class of phytochemical compounds that affect the pro-coagulation of blood in an extract.[26] Tannins were known as an adstringent agent by increasing the vasoconstriction process in small blood vessels which is an important parameter in hemostasis, so tannins can be useful as hemostatics and reduce bleeding time. Beetroot is known as contains vitamin K.[27] Vitamin K is a blood coagulation cofactor that can reduce bleeding time.[28] Vitamin K acts as a cofactor for several coagulation factors in the intrinsic and extrinsic coagulation pathways.[29] vitamin K is an important cofactor for the synthesis of factor II, factor VII, factor IX, and factor X so with its various components, beetroot has benefits in reducing bleeding time.[30] Based on the data in tables 4-6 it can be observed that there is dose-dependent activity. Along with the increase in the tested dose of beetroot extract, the effect of beetroot ethanol extract also increased in shortening the bleeding time in heparin-induced rats. Beetroot extract has a compound that functions to increase the platelet count. The results of statistical testing of platelet count at several variations in the increase in the dose of beetroot extract at the dose of 100 mg/kg BW, 200 mg/kg BW, and 400 mg/kg BW, were statistically significant ($p <0.05$) against the negative control group that was induced by heparin, this is stated that beetroot extract administration can increase the platelet count of heparin-induced rats. Several studies have indicated the quercetin content in beetroot.[31,32] The quercetin has an effect on increasing granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) which will stimulate megakaryopoiesis which will increase the platelet count.[33] The results of statistical testing of the prothrombin time value at several variations in the increase in the beetroot extract dose at the dose of 100 mg/kg, 200 mg/kg, and 400 mg/kg BW, were statistically significant ($p<0.05$) against the negative control group induced by heparin. This suggests that the administration of beetroot extract can reduce the PT value of heparin-induced rats. Beetroot has been reported to contain vitamin K.[27] The occurrence of prolonged PT is a sign of a disruption in the extrinsic coagulation pathway in factors VII, X, V, and II.[34] Along with the increase in the treatment dose of beetroot extract, the effect of ethanol extract of beetroot was also increased in shortening the prothrombin time of heparin-induced rats. All of the tested doses showed a dose-dependent manner.

CONCLUSION

An ethanol extract of beetroot (Beta vulgaris L) has an overall phenolic and flavonoid concentration of 312.2 mg GAE/g extract and 38.5 mg quercetin equivalent/g, respectively. It exhibits hemostatic capabilities as well as a dose-dependent behaviour.

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[RJC-6728/2021]