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Research article

In vitro hemostatic activity of ethanol extracts of Beetroot (*Beta vulgaris* L.) in blood male albino rat

Sony Eka Nugraha*, Edy Suwarso, Yuandani

Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia.

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***Corresponding Author: Sony Eka Nugraha,** Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia.

Abstract

Objective: The purpose of this research was to determine the in vitro hemostatic activity of ethanol extract of beetroot (*Beta vulgaris* L). **Methods:** In vitro hemostatic activity was performed on whole blood of rat by Lee-White method to determine the clotting time and the Eustrek method to observe the microscopic picture of clotted blood. **Results:** The results of in vitro study showed that ethanol extract of Beetroot at the concentration of 1% and 2% decreased the clotting time at the minute of 24.4±1.14 and 15.9 ± 0.65, respectively, as compared to EDTA treatment which did not clot for 120 min ($p<0.05$) and microscopically showed that blood cells appear to be attached to each other at the concentration of 1% and 2% of ethanol extract of Beetroot. The hemostatic activity of ethanol extract of beetroot showed a dose-dependent manner in the in vitro study. **Conclusion**: Ethanol extract of beetroot (*Beta vulgaris* L) has hemostatic activity on in vitro method.

Introduction

Hemostasis is the process of blood clot formation and represents a coordinated response to vessel injury [1]. It is accomplished by the coordinated efforts of 3 distinct but intimately related mechanisms: the vascular, the platelet, and the coagulation phases of hemostasis [2].

Bleeding disorders are characterized by defects in hemostasis that lead to an increased susceptibility to bleeding. They are caused either by platelet disorders, coagulation defects or, in some cases, a combination of both [3].

Indonesia has many plants that can be used as sources of medicinal ingredients since a long time ago. There are many plants commonly used for healing hemostatic disorders such as red guava, dates, papaya and beetroot. Indonesian believed that beet root (*Beta vulgaris,* L) may increase the number of platelets and improve the recovery of dengue fever patient.

Beetroot contains of some secondary metabolite compounds such as tannin saponins, alkaloids, flavonoids, terpenoids and steroids. Some minerals are also contained in beetroot such as iron (Fe), magnesium (Mg), copper (Cu), sodium (Na), potassium (K), mangan (Mn), calcium (Ca) and zinc (Zn) [5].

Calcium has been identified as coagulation factor on the coagulation cascade.Calcium could accelerate the formation of thrombin and stimulate the formation of fibrin [6].

According to previous research, methanol extract of beetroot has benefits in improving formation and development process of blood cells which have effect to hemostatic process [7].

In the present study the ethanol extracts of beetroot were investigated for its hemostatic effects by determining the blood clotting time and microscopic examination.

Material and methods

Material

Ethanol 96%, aqudest, Ethylene Diamine Tetraacetic Acid (EDTA) and Diethyl Ether (Bratachem, Indonesia).

Plant collection

Beetroot tuber was collected from local market at Padang Bulan, Northern Sumatra, Indonesia. The plant samples authenticated by Research Center of Biology, Indonesian Institute of Science, Bogor, Indonesia.

Extraction of beetroot

An amount of 300 g dried material plant samples were crushed in a blender, then macerated in ethanol 96% for 3 hours thereafter moved to perlocator tube. Percolation was stopped if the last 500 mg of solvent were evaporated, leaving no residuals. The solvent was evaporated at low pressure with a temperature of not more than 40°C using a Rotary evaporator.

Phytochemical screening of ethanol extract of beetroot

Phytochemical screening carried out on ethanol extract beetroot includes examining the chemical secondary

metabolites of alkaloids, flavonoids, glycosides, tannins, saponins, triterpenoids, and steroids [8-10].

Animals and blood sample

Animals used in this study were 5 male Wistar rats, weighing 180-220 g. The blood sample was collected from sinus retro orbital.

In vitro hemostatic activity test

In vitro hemostatic activity was performed on whole blood of rat by Lee-White method to determine the clotting time and Eustrek method to observe the microscopic picture of clotted blood [11].

Determination clotting time by Lee-white method

The rats were divided into 5 groups then anesthetized using diethyl ether. Blood was collected from sinus retro orbital 2.5 ml. Each group consists of 5 tubes. Normal group contain of 0.5 ml blood, negative control group contain of 0.5 ml EDTA 15% then added 0,5 ml of blood. Treatment group consisted of the addition 0.5%, 1% and 2% beetroot extracts.

At the time of adding blood into each tube, the stopwatch is run to determine the clotting time. Every 30 seconds, each tube are tilted and the blood clot is observed until coagulation or clot occurs for 2 hours.

Microscopic examination by Eustrek method

Five pieces of object glass were prepared. At the end of lee white method test, one drop of blood was taken from each group and put it in object glass. Using the corner of another object glass, spread the blood drop. The blood clot was observed under 40 magnification microscope.

Statistics

Analysis of all results was performed using ANOVA with Tukey's Multiple Comparison Test. *P* values for significance were set at 0.05. Values for all measurements are expressed as the mean ± SD.

Results

Phytochemical screening result of ethanol extract of beetroot

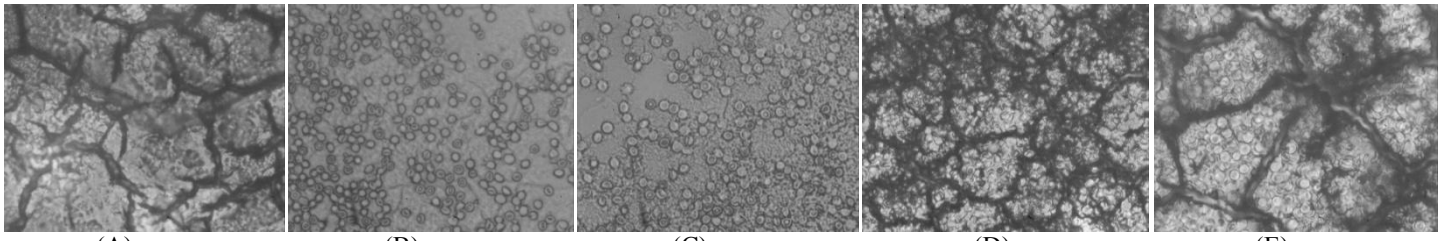
Phytochemical screening result showed that ethanol extract of beetroot positively contains flavonoids, alkaloids, saponins, tanins, glycosides and steroids/triterpenoid.

Determination clotting time

Determination clotting time by Lee-white method showed that Beetroot extracts of 1% and 2% could significantly shorten the clotting time. The clotting time of the blood from all groups are shown in Table 1.

Microscopic examination

The results of microscopic examination of blood clot are shown in Figure 1.



(A) (B) (C) (D) (E)

Figure 1. Representation microscopic examination of (A) normal blood, (B) negative control, (C) 0.5 % extract, (D) 1 % extract, (E) 2 % extract.

Table 1. In vitro blood clotting time

Group	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Clotting time (min) Mean ± SD
Normal (blood 0.5 ml)	4.5	4.5	5	4.5	5	4.7 ±0.27*
Negative control: (blood 0.5 ml + EDTA 15% 0.5 ml)	120	120	120	120	120	120
Extract 0.5% 100µl + EDTA 15% 0.5 ml + blood 0.5 ml	120	120	120	120	120	120
Extract 1% 100 µl + EDTA 15% 0.5 ml + blood 0.5 ml	25.5	23.5	23	24.5	25.5	24.4 ± 1.14*
Extract 2% 100 µl + EDTA 15% 0.5 ml +blood 0.5 ml	15.5	15.5	16	17	15.5	15.9 ± 0.65*

(Mean ± SD, n = 5). Significance of differences with negative control: * P < 0.05.

Discussion

The ethanol extracts of beetroot at a concentration of 1% and 2%, significantly shorten the clotting time. Blood clotting time in normal group was determined with an average time of 4.7 ± 0.27 min. The negative control group did not clot after being observed for 120 min. EDTA is an anticoagulant that works by binding calcium, which is one of the coagulation factors of blood [12]. Blood clots did not appear in the negative group because the calcium has been bound by EDTA.

The blood after addition of 0.5% beetroot extract did not clot for 120 min observation. EDTA is used as a chelating agent that binds to calcium, which is a coagulation factor [13]. Insufficient concentration of beetroot extract involve extract could not block the metal and mineral chelation mechanisms by EDTA.

Blood clotting time in group of addition 1 % beetroot extract was determined with an average time 24.4 + 1.14 minutes. Treatment group of 1% extract is able to work as a coagulant even with EDTA because the mineral content in the beetroot extract could block the EDTA chelation mechanism in the blood.

EDTA has 6 multidentate ligands of 4 Oxygen atoms and 2 Nitrogen atoms that can form chelates with various mineral and metal compounds [11]. Beetroot contains several minerals such as iron (Fe), Magnesium (Mg), Copper (Cu), Sodium (Na), Potassium (K), Mangan (Mn), Calcium Ca) and Zinc (Zn) [5]. EDTA was binding those minerals and decrease the effect in preventing blood clots. Treatment group of 2% extract has stronger activity in shorten the clotting time rather than 1% extract due to higher concentrations that induce faster coagulation effect than 1%. The hemostatic activity ethanol extract of beetroot showed a dose-dependent manner in the in vitro study.

According to Figure 1 (A), microscopic examination of normal blood showed that blood cells lysis and appear to be attached each other and forming groups. Attached cells in clotted blood can cause cell wall to be lysis and has no more shape [15].

Figure 1 (B) showed that EDTA addition prevent blood to clot and blood cells appear in normal shape. Blood clotting can be prevented by the addition of chelating agent such as EDTA by binding calcium ions which is a coagulation factor of blood [13].

Figure 1 (C) showed that the addition of 0.5% beetroot extract did not clot the blood cells. In line with the clotting time determination, insufficient concentration of beetroot extract involve extract could not block the metal and mineral chelation mechanisms by EDTA.

Figure 1 (D) showed that the addition of 1 % beetroot extract could block the EDTA chelation mechanism. Cells appear to be attached to each other and forming groups. Some cells appear in normal shape and there are some cells become lysis. Figure 1 (E) has a similar representation to Figure 4, addition of 2 % beetroot extract also block the EDTA chelation mechanism.

Conclusion

Ethanol extract of beetroot at the concentration of 1% and 2% decreased the clotting time as compared to the negative control group. Blood cells after introduced by 1% and 2% extracts has clotted which characterized by blood cells appearing attached to each other when observed microscopically

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