

Effect of Broccoli (*Brassica oleracea* L. var. *italica*) Extract on Bleeding Time in Male White Mice (*Mus musculus* L.)

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Abstract

Cessation of bleeding is influenced by many factors including the type of medication used. The use of drugs to stop bleeding can be done in various ways, one of which is the use of traditional medicine. One of the traditional plants that can be used as a stop bleeding is broccoli (*Brassica oleracea* L. var. *italica*) which has a fairly high vitamin K content, where is efficacious as hemostatic. This study aims to determine the effect of broccoli on the bleeding time of mice tail cuts. The first group was negative control (Na-CMC), the second group was positive control (tranexamic acid), while groups three, four, and five were broccoli extract dose of 20, 40, and 60 mg/kg BW, respectively. All treatments were given orally for seven days. Bleeding time was calculated from the initial blood loss until the blood stopped (tail bleeding I method) and the data were analyzed with the ANOVA one way test and the post hoc Least Significant Difference (LSD) statistical test. The results showed that there were differences in the time to stop bleeding in the treatment control group at a dose of 20 mg/kg BW (122.60 ± 29.535 seconds), 40 mg/kg BW (102.40 ± 9.607 seconds) and 60 mg/kg BW (90.40 ± 3.845 seconds). From these results, it can be concluded that the extract of broccoli at a dose of 60 mg/kg BW gives the best results as hemostatic, while the effect is almost similar to the positive control group.

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INTRODUCTION

External bleeding is bleeding originating from an open wound so that it can be seen from a physical examination (Guo & DiPietro, 2010). Bleeding is a process of bleeding from blood vessels that can cause damage to blood vessel walls caused by trauma or disease (Xu *et al.*, 2019). The normal functioning hemostatic system is important for the life of the organism because if the hemostatic is disrupted, even a small wound can cause life-threatening bleeding (Periayah *et al.*, 2017).

The normal innate hemostatic mechanism of the body is sufficient to repair damage and stop the secretion of blood from these delicate microcirculation vessels (Chen *et al.*, 2018). The body's mechanism in stopping bleeding

involves three main steps, including vascular spasm, formation of platelet plugs, and blood coagulation (formation of blood clots) (Sherwood, 2015). Hemostatic failure causes bleeding and is a dangerous clinical problem (Pierce & Pittet, 2014).

An example of a well-known failure of the hemostatic process is hemophilia. Hemophilia is a very common disease which refers to the tendency to experience severe excessive bleeding (Curnow *et al.*, 2016). In the United States, about 1 in 10,000 people suffer from hemophilia with severe severity. From all the case, four out of five cases are caused by factor VIII deficiency (Sacher, 2012).

Wound healing is influenced by many factors including the type of medication used. The use of drugs for wound

healing can be done with a variety of types and types, one of which is the use of traditional medicine (Guo & DiPietro, 2010; Saghadzadeh *et al.*, 2018). The use or treatment is traditionally increasingly preferred because in general there are fewer side effects as well as drugs from chemicals (Yuan *et al.*, 2016).

Broccoli (*Brassica oleracea* L. var. *italica*.) is a family of Brassicaceae that contains good phytochemicals such as glucosinolates, phenolic compounds, fiber and antioxidant compounds such as vitamins C and E and minerals (Ca, Mg, Se, and K) (Raiola *et al.*, 2017). According to United States Department of Agriculture (2012), compared with other vegetables including carrots, cabbage, and spinach, the vitamin K content in broccoli is higher at 101.6 mg or 85% greater than other vegetables. Based on this background, this study aims to determine the effect of broccoli on the bleeding time of mice tail cuts.

MATERIALS AND METHODS

Materials and tools

The material used in this study were broccoli, tranexamic acid (Kalnex®), Na-CMC, natrium chloride 0.9% (Otsu®), ethanol, concentrated hydrogen chloride, Dragendorff reagent, Mayer reagent, 10% iron (III) chloride, amyl alcohol, chloroform, anhydrous acetic acid, and male white mice (*Mus musculus* L.) weighing between 20 and 30 g. The tools used in this study include glassware (Iwaki®), hot plate, oven, vacuum rotary evaporator (Heidolph®), analytical scale (Kenko®), stirring rod, stopwatch, and measuring flask (Iwaki®).

Extracting preparation

Broccoli used in this research was 5 kg of fresh broccoli obtained from Samarinda market in Batam City, which has been identified in Herbarium of Universitas Andalas, Padang. Broccoli that has been prepared is then washed using running water. After washing, broccoli is cut into small pieces. Broccoli chunks are then dried in the

morning sun for three consecutive days. After drying, broccoli is extracted by maceration method using 70% ethanol for three days while stirring occasionally. The extract obtained was then evaporated using a vacuum rotary evaporator at 40°C to obtain a thick extract.

Phytochemical screening

Alkaloid test

A total of 2 ml of the extract solution was evaporated in a porcelain cup. The residue obtained is then put into a test tube and 5 ml of 2 N HCL is added. The solution is then divided into 2 tubes. Tube 1 is added 2-3 drops of Dragendorff reagent, while tube 2 is added Mayer reagent. Positive results of the alkaloid content are indicated by the formation of red brick, red, or orange colors with the Dragendorff reagent, and white or yellow deposits with the Mayer reagent (Auwal *et al.*, 2014).

Saponin test

As much as 0.5 g of broccoli extract is added to 0.5 ml of hot water. Cool the mixture first to room temperature then shake vigorously for 10 seconds to produce solid foam as high as 1-10 cm. Then 1% HCl is added and waited for 10 minutes. Positive results from the saponin content are shown if the foam does not disappear (Hossain *et al.*, 2013).

Tannin test

Broccoli extract is boiled with 20 ml of water then filtered with filter paper and then added a few drops of 10% FeCl₃. A positive result of the tannin content is shown if the solution produces a greenish brown or black-blue color (Batoool *et al.*, 2019).

Flavonoid test

As much as 0.5 g of broccoli extract is added to a small amount of Mg powder and then shaken until mixed. The mixture is then added with a few drops of concentrated HCl. The positive results of flavonoids are marked by the formation of orange, red, or yellow (Panche *et al.*, 2016).

Animal care and handling

Test animals used in this study were Swiss Webster male mice selected by purposive sampling with age requirements of around 2-3 months with a body weight of about 20-30 g. The selection of test animals is done by simple random sampling (Samanta *et al.*, 2016). Determination of the sample size of each group is determined based on the Federer calculation formula obtained at least five mice per group for a total of five test groups. The total number of test animals to be used is 25 male mice. Before giving treatment, the test animals were acclimatized for seven days. The treatment of test animals is carried out based on research code of ethics using test animals with protocol number 3404012S121242020022800279 issued by Universitas Aisyiyah Yogyakarta.

Provision of test treatment

After going through the acclimation process, test animals are given treatment based on each test group. The negative control group was given 0.5% Na-CMC, the positive control group was given tranexamic acid as much as 102.74 mg/20 g BW. While treatment groups I, II and III, each given a dose of broccoli extract of 20 mg/kg BW, 40 mg/kg BW, and 60 mg/kg BW, respectively.

Bleeding time

Determination of bleeding time is done by calculating the time needed starting from the wound starting to drip blood until the blood stops dripping from the wound. First the rats' tails were cleaned with 70% alcohol then cut 1 cm from the tail end. The cut tail is inserted into a tube containing warm NaCl (37°C). The duration of bleeding is calculated using a stopwatch from the onset of blood droplets from injured blood vessels until the blood stops flowing out of the blood vessels (Liu *et al.*, 2012). The time interval from the first drop until the blood stops dripping is the bleeding time.

Data analysis

The data obtained were analyzed using SPSS version 21. To determine the normality of data distribution, tests were performed using the Shapiro-Wilk test. Homogeneity test was performed using the Levene variant test. Then the Least Significant Difference (LSD) test is performed to see the differences between each treatment group.

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical screening results of broccoli extract indicate that positive broccoli plants contain alkaloids, saponins, tannins, and flavonoids. These results are in line with several previous studies which showed the existence of the compound class (Chauhan *et al.*, 2016; Hussain *et al.*, 2019; Mageney *et al.*, 2017; Raiola *et al.*, 2017). The complete phytochemical screening results are presented in **Table I**.

Table I. Phytochemical screening results

Phytochemical	Reagent	Result	Conclusion
Alkaloid	Dragendorff	Orange precipitate	+
	Mayer	White deposit	+
Saponin	Water + HCl	Foaming over 10 seconds	+
Tannin	FeCl ₃	Blackish brown	+
Flavonoid	Mg + HCl	Yellow	+

Bleeding time

The parameter observed in this test is the bleeding time. The average measurement of bleeding time in the positive control group was 73.80 ± 6.76 seconds, while in the negative control was 183.00 ± 18.193. The bleeding time of the positive control group was shorter than the negative control group due to the administration of tranexamic acid which served as a coagulation agent. Tranexamic acid works as an anti-fibrinolytic agent by

inhibiting the breakdown of polymer fibrin by plasmin, so that hemostasis can occur more effectively (Levy *et al.*, 2018). The treatment group of Broccoli extract dosages of 20, 40, and 60 mg/kg BW yielded the averaging time of 122.60 ± 29.535 ; 102.40 ± 9.607 ; and 90.40 ± 3.845 seconds, respectively. Comparison of bleeding time for all groups is presented in **Table II**, while the comparison for average bleeding time is presented in **Figure 1**.

Table II. Bleeding time for all groups

Group	Bleeding time (s)/mice					Mean \pm SD
	1	2	3	4	5	
Negative control	163	202	164	192	194	183.00 \pm 18.193
Positive control	65	73	84	73	74	73.80 \pm 6.76
Broccoli Extract 20 mg/Kg BW	102	122	173	101	115	122.60 \pm 29.535
Broccoli Extract 40 mg/Kg BW	93	102	93	110	114	102.40 \pm 9.607
Broccoli Extract 60 mg/Kg BW	85	90	89	93	92	90.40 \pm 3.845

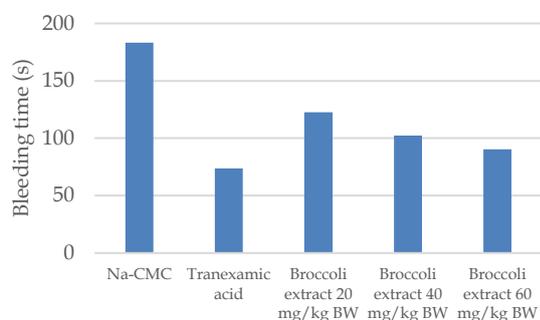


Figure 1. Comparison of average bleeding time for all test groups

This result indicated that bleeding time of the broccoli extract treatment group was shorter than the negative control. This is due to the presence of substances in the extracted content of broccoli which functions as a coagulation consisting of vitamin K (Janarthanan & Kumar, 2013). Vitamin K has an important role in clotting physiology, including as a cofactor for carboxylation of glutamate residues in post-synthesis modification of

proteins to form unusual carboxyglutamate amino acids (Rishavy & Bekner, 2012).

Bleeding time was observed to see the effect of the test material on the formation of temporary coagulation plugs, namely platelet phase hemostasis (primary hemostasis). This will occur if there are desquamation and small injuries to the blood vessels. Primary hemostasis involves the intima of blood vessels and platelets. Wounds will induce vasoconstriction and platelet plugs. The time from the start of the wound to the formation of a temporary coagulation plug in the wound area is called the bleeding time. The effect is shown by the shorter bleeding time after giving test material (Periayah *et al.*, 2017).

Statistical analysis

Determination of the normality of data distribution on the percentage of stopping bleeding is done using the Shapiro Wilk test. The analysis showed that the data were normally distributed ($p > 0.05$). The test was then continued with a variant homogeneity test for the percentage of cessation of bleeding using the Levene test. The results obtained indicate a significance value ($\text{sig.} > 0.05$) so that the percentage of data obtained is declared homogeneous. The test then continued with the one-way ANOVA test.

Percentage data of bleeding cessation show significance value $p < 0.05$. These results indicate that there are differences between each treatment. This indicates that there was a process of stopping bleeding in the rat's tail due to treatment with broccoli extract and positive control. The test then continued with post hoc using the LSD test to see the comparison of differences between treatment groups in more detail.

The positive control group and broccoli extract with concentrations of 40 and 60 mg/kg BW showed a significant difference in stopping bleeding ($p < 0.005$) compared to the negative control. While broccoli extract

with a concentration of 20 mg/kg BW did not show any significant difference compared to negative controls. There were significant differences between positive control with the broccoli extract group 20 and 40 mg/kg BW ($p < 0.005$), while in the 60 mg/kg BW group there was no significant difference ($p > 0.005$). These results indicate that broccoli extract with a concentration of 60 mg/kg BW is not so different when compared to the tranexamic acid in reducing bleeding time. These results reinforce the notion that broccoli extract can be an alternative to be considered as a substitute for tranexamic acid as a hemostatic agent. The LSD test results from each group are presented in **Table III**.

Table III. Post Hoc LSD test results for each test group

Comparison Group		Sig	Conclusion
Negative Control Group (Na-CMC)	Positive Control Group	0.000	Different
	Extract 20 mg/Kg BW	0.006	No different
	Extract 40 mg/Kg BW	0.000	Different
Positive Control Group (Tranexamic acid)	Extract 60 mg/Kg BW	0.000	Different
	Negative Control Group	0.000	Different
	Extract 20 mg/Kg BW	0.000	Different
Broccoli Extract 20 mg/Kg BW	Extract 40 mg/Kg BW	0.012	Different
	Extract 60 mg/Kg BW	0.127	No different
	Negative Control Group	0.006	No different
Broccoli Extract 40 mg/Kg BW	Positive Control Group	0.000	Different
	Extract 40 mg/Kg BW	0.003	Different
	Extract 60 mg/Kg BW	0.006	Different
Broccoli Extract 60 mg/Kg BW	Negative Control Group	0.000	Different
	Positive Control Group	0.012	Different
	Extract 20 mg/Kg BW	0.003	Different
Broccoli Extract 60 mg/Kg BW	Extract 60 mg/Kg BW	0.010	Different
	Negative Control Group	0.000	Different
	Positive Control Group	0.127	No different
Broccoli Extract 60 mg/Kg BW	Extract 20 mg/kg BW	0.006	Different
	Extract 40 mg/kg BW	0.010	Different
	Extract 60 mg/kg BW	0.010	Different

CONCLUSION

Based on the results of this study it can be concluded that the extract of broccoli at a dose of 60 mg/kg BW gives the best results as hemostatic, while the effect is almost similar to the positive control group. Further study needed for further observe the wound healing effect of broccoli extract and from its active metabolites.

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