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ORIGINAL RESEARCH

Estimation of Platelet Count and Bleeding Time of Mice Treated with *Musa paradisiaca* var. sapientum (L.) *Kuntze* Extract

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Objective: The aim of this study was to estimate the platelet count and bleeding time on peripheral blood smear of mice tail wound using *Musa paradisiaca* var. sapientum (L.) *Kuntze* (ambonese banana stem extract).

Design: Randomized post-test-only control group design.

Materials and Methods: Twenty-four male mice (Mus Musculus) were randomly divided into 4 groups. A negative control group was treated with carboxymethyl cellulose (CMC), a positive control group (K+) treated aspirin 100 mg/kg body weight, group P1 treated with aspirin 100 mg/kg body weight and tranexamic acid 50 mg/kg body weight, and group P2 treated with 30% of ambonese banana stem extract (ABSE). The mean and standard deviation data of platelet counts and bleeding time were analyzed by one-way ANOVA statistical software.

Results and Discussion: Tranexamic acid had no significant effect on platelets count compared to CMC group ($p = 0.871$), but administration of aspirin resulted in low platelets count significantly ($p = 0.003$). The platelet counts of ABSE and CMC groups were not significant different ($p = 0.937$). Aspirin has significantly shown prolonged bleeding time than CMC, tranexamic acid, and ABSE groups. However, there was no difference between the tranexamic acid and ABSE groups ($p=0.934$). The bleeding time of tranexamic acid and ABSE groups was similar, although the platelet count in the ABSE group was lower than in the CMC group.

Conclusion: This study proved that ambonese banana stem extract has a potency to shorten the bleeding time in mice tail wound without interfering to platelet count.

Keywords: haemorrhage, health risk, herbal medicine, peripheral blood smears, wound

Introduction

Wound is the loss or damage of some body tissues caused by a sharp or blunt trauma, temperature changes, chemicals, burning, electric shock, or animal bites. This damage causes significant morbidity rates worldwide; therefore, its management is needed to effectively facilitate the normal healing process for quick recovery.[1–3] Open wounds on the body surface cause rupture of blood vessels leading to bleeding. In mild cases, the haemorrhage stops quickly, but when it is wide and deep enough, it takes longer time to stop.[4] A severe bleeding can cause impaired perfusion, leading to tissue hypoxia which can cause hypovolemic shock.[5] This condition is due to insufficient blood in the circulation. Therefore, hypoperfusion (low oxygen perfusion) disrupts cell metabolism, and reduces adenosine triphosphate (ATP) formation causing general organ failure.[6]

Post-extraction teeth haemorrhage can cause severe complications such as uncontrolled bleeding and infection. Wounds after tooth extraction should be well dried because there are various kinds of infectious bacteria in the oral cavity.[7,8] Blood clotting is a series of complex process to achieve hemostatic state that prevents excessive blood loss after

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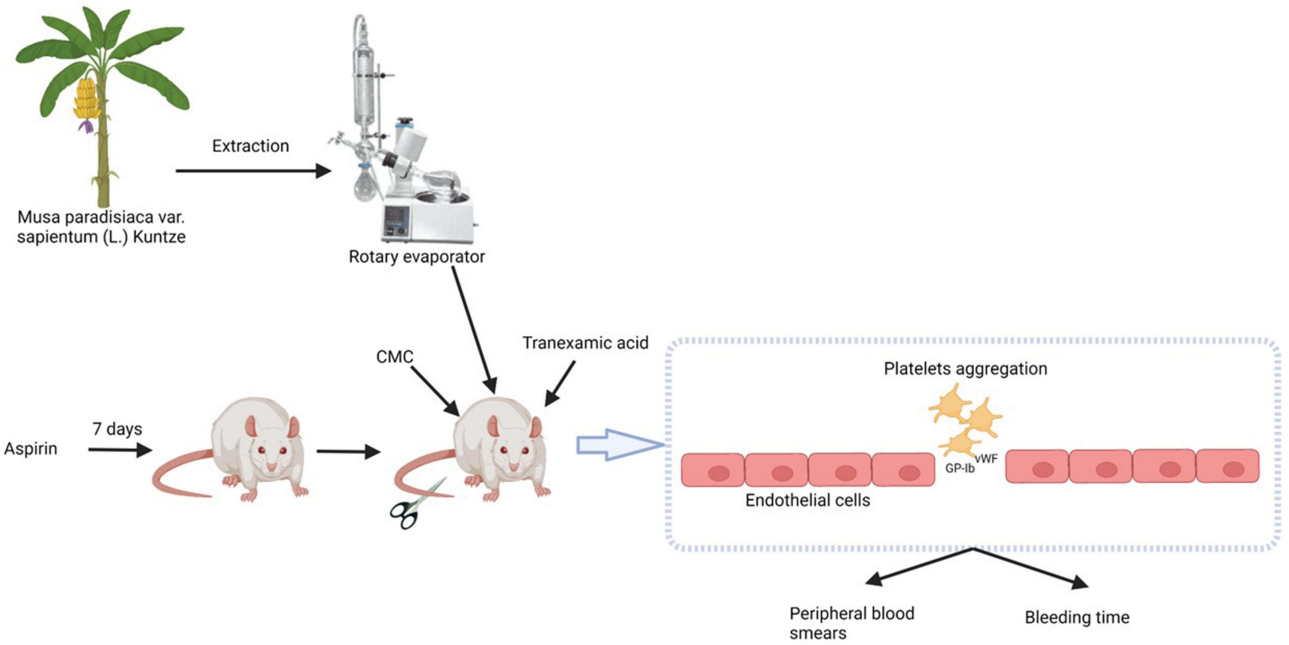
Graphical Abstract



injury.[9] When bleeding occurs, there are three main processes of hemostasis and coagulation which includes vasoconstriction, platelet aggregation, and activation of blood clotting factors. Therefore, platelets have a major role in the hemostatic phase by forming blood plugs and activating the clotting factors.[10,11] Hence, the significance of hemostatic analysis to estimate platelet function, which could be done on a peripheral blood smear under a light microscope.[12,13]

However, severe haemorrhage can occur in patients who consume blood-thinning aspirin.[14] Hence, adequate bleeding control can be done by administering systemic hemostatic drugs such as tranexamic acid.[15] Several studies have shown that the administration of hemostatic drug in postoperative patients can have side effects such as nausea, vomiting, headaches, and allergic reactions.[16] In fact, these medications are contraindicated in stroke patients. Because the usage of existing coagulants is associated with evident side effects, new coagulants should be created with a focus on medicinal plants that have relatively few adverse effects.[17]

There are several plants with medicinal properties which can be used to heal wounds and prevent infections with minimal side effects, therefore they are applied as therapeutic drugs. Besides, it has been found that those plants with active ingredients can play an important role in wound healing. In Indonesia, banana trees have been proven to contain saponins, flavonoids, alkaloids, tannins and lectins which have various health benefits such as antihypertensive and antidiabetic effects. Furthermore, ambonese banana sap has various therapeutic effects as an antioxidant, antibiotic, and wound healing accelerator.[18] The application of ambonese banana stem sap at a concentration of 30% accelerated wound healing by increasing the expression of platelet-derived growth factor (PDGF-BB).[19] Effects of PDGF-BB topical treatment in animal models have been shown to improve tissue repair under conditions of delayed wound healing.[20] Therefore, the aim of this study was to estimate the platelet count and bleeding time on peripheral blood smear of mice tail wound using Ambonese banana stem extract (ABSE) as a medicinal plant in hemostasis.

Materials and Methods

Samples and Ethical Clearance

This study has passed the ethical clearance of health research no. 586/HRECC.FODM/IX/2019 Faculty of Dental Medicine, Universitas Airlangga. In compliance with Government Regulation of the Republic of Indonesia Number 95

of 2012 Concerning Veterinary Public Health and Animal Welfare. The procedures and methods that have been prepared based on the consensus of all parties concerned with due regard to the requirements for safety, security, health, environment, development of science and technology, and experience of both current and future developments in order to obtain the maximum benefit possible. We used 24 male mice (*Mus Musculus*), weighing between 20 and 30 g body weight (BW). Sample size formula was obtained from health studies formulation as amount 6 samples each group. In the negative control group (K-), the mice were given carboxymethyl cellulose (CMC) orally. Meanwhile, the positive control group (K+) was given 100 mg/kg body weight aspirin dissolved with 1% CMC orally for 7 days. The treatment group-1 (P1) was given aspirin dose of 100 mg/kg body weight dissolved with 1% CMC orally for 7 days and given 50 mg/kg body weight of tranexamic acid orally 30 minutes before cutting the tail. The treatment group-2 (P2) was given aspirin dissolved in 1% CMC orally for 7 days and given ABSE at concentration of 30% 30 minutes before cutting the tail. The solution volume given orally was 1 mL/10 g body weight.

Preparation of Ambonese Banana Stem Extract (ABSE)

The stems were obtained from the Plant Conservation Center in Purwodadi Botanical Gardens, at Pasuruan. The plant was identified at LIPI (Indonesian Institute of Sciences) no. 1272/IPH.06/HM/XI/2019, which confirmed it as ambonese banana. The selected plant was 2.5–3 m high with a diameter of around 17–18 cm. Furthermore, the age was between 12 and 13 months. The identified stem sap was used according to the previous study.[19]

The stem sap was taken by cutting the bottom and then washing it to remove dirt. Afterwards, they were cut into small pieces with a sterile knife, and distilled water of 200 mL was added till they blend.[21] A homogeneous banana stem sap and aquades were filtered using a bugner funnel connected to a vacuum pump (Gast brand, USA) and given whatman filter paper number 41. The resulted extract was stored in a closed dark bottle to reduce oxidation.[22]

Platelets Count Analysis on Peripheral Blood Smears

Blood samples are taken through a scalpel cut near the tail tip. The last drop of blood was rubbed on a glass object. The platelet count based on the results of peripheral blood smears was then calculated using a counting chamber. The smear was stained with Giemsa (Sigma-Aldrich, USA) and observed in five visual fields with a 400x magnification microscope (Nikon H600L, Japan). The blood was placed at a distance of 2–3 mm from the end of the glass. Other smears were made with a different glass and placed at an angle of 30–45 degrees in front of the blood droplets. The smears were left to dry and labeled according to the treatment group. Giemsa dye was diluted with buffer (1:4), then colors were mixed with methanol until it flooded the preparation. The results were then observed in five visual fields under a microscope at 400x magnification.[23]

Tail Bleeding Time Analysis

Bleeding time test was performed on experimental animals. The mice's tail was cleaned with 70% alcohol and cut at a point of 2 cm from the tip using surgical scissors. The dripping blood was absorbed on whatman absorbency paper. Meanwhile, the time from the first blood drip until it stopped was calculated as the bleeding time. Furthermore, the blood was allowed to drip spontaneously and placed on 16 absorbent paper boxes every 15 seconds.[24]

Statistical Analysis

The data were analyzed using *Statistical Package for the Social Science Software* (SPSS). It was presented in the form of *mean ± standard deviation* ($\overline{X} \pm SD$). In addition, the normality test of platelet count and bleeding time data used *one sample Kolmogorov–Smirnov*, while the homogeneity used *Levene Test*. The mean difference between groups was analyzed using *a one-way ANOVA* test and *least significant difference (LSD)* at 95% confidence interval.

Results

The data were presented in the form of figures, tables representing mean ± standard deviation ($\overline{X} \pm SD$), and graphs. Furthermore, statistical analysis was used to test the hypotheses.

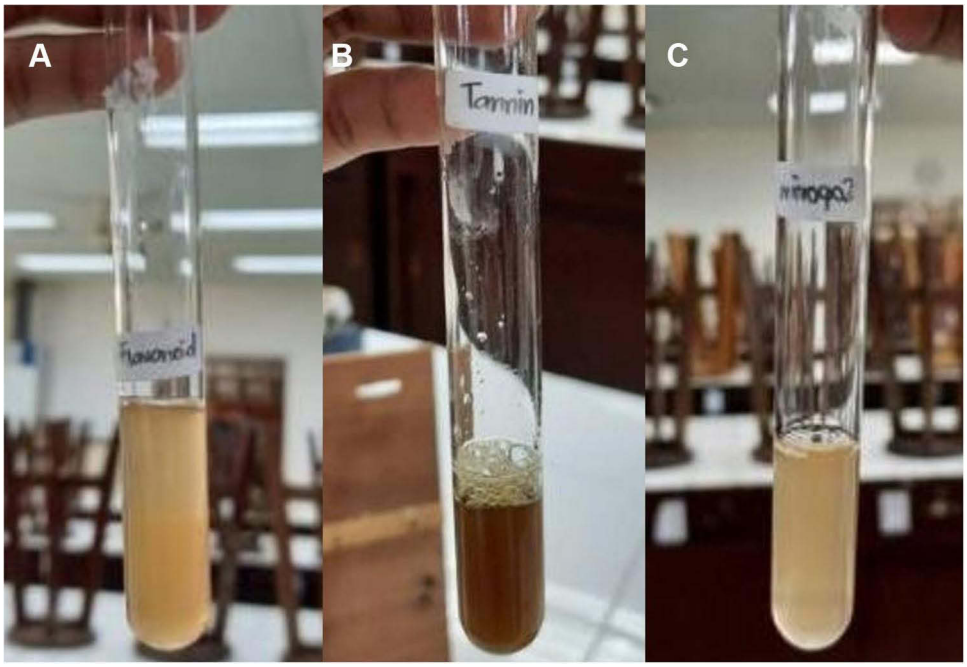


Figure 1 Phytochemical analysis of ambonese banana stem extract content. (**A**) Flavonoid, (**B**) Tannin and (**C**) Saponin.

Phytochemical Analysis of Ambonese Banana Stem Extract

We have provided the qualitative phytochemical analysis on the active substances in the stem extract. Positive results were obtained from the flavonoid, tannin and saponin tests. The flavonoid test was positive because the solution color changed to yellowish-orange. Moreover, the tannin test was positive because there was a color change to dark-green after adding FeCl3 reagent. Furthermore, the saponin test was positive because there was visible foam formation that was stable for about 10 minutes (Figure 1).

Analysis of Platelets on Peripheral Blood Smears

The mean of platelet count from 24 mice samples showed 163.3 in the CMC group, 78.0 in the aspirin group, 169.7 in the tranexamic acid group, and 163.0 in the ABSE group. Based on these data, the application of tranexamic acid and ABSE increased platelets around the wound compared to the aspirin group, and equal to the CMC group (negative control) (Table 1).

According to Table 1, there was a significant difference between groups in platelet count ($p = 0.000$). Tranexamic acid had no significant effect on platelets count compared to the CMC group ($p = 0.871$), but administration of aspirin resulted in low platelets count significantly ($p = 0.003$). The platelets count of ABSE and CMC groups were not significant

Table 1 The Mean and Standard Deviation of Platelet Count in Peripheral Blood Smears

Groups	Platelet Count $\overline{X} \pm SD$ (10^3 Cells)
(K-) CMC	163.3 ± 2.4
(K+) Aspirin	78.0 ± 1.2*
(P1) Tranexamic Acid	169.7 ± 2.8
(P2) Ambonese Banana Stem Sap	163.0 ± 2.9

Note: *Indicated significantly different ($p < 0.01$).
Abbreviations: $\overline{X}$, mean; SD, standard deviation.

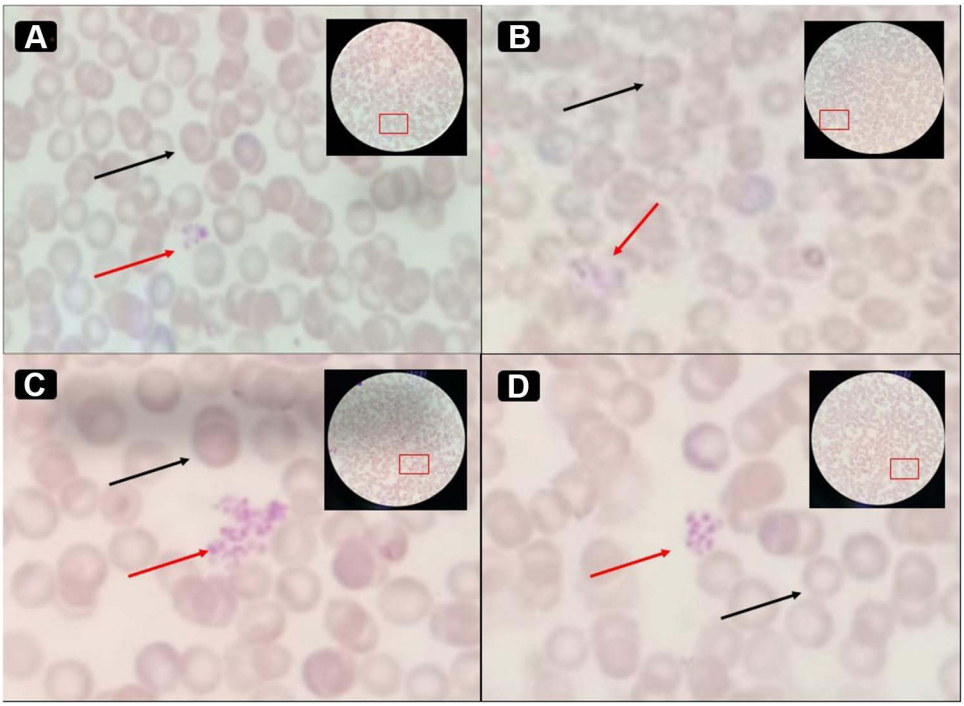


Figure 2 The platelet count in a peripheral blood smear with Giemsa staining. (**A**) CMC, (**B**) Aspirin, (**C**) Tranexamic acid, (**D**) Ambonese banana stem extract. Black arrow: red blood cells; Red arrow: platelets.

different (p = 0.937). The peripheral blood smear with Giemsa staining was examined and counted under a light microscopy (400×) (Figure 2).

Effect of ABSE on the Tail Bleeding Time

The bleeding time of CMC group was 115 sec, and aspirin group was 232.5 sec. It means that the bleeding time of the aspirin group was longer than that of the negative control group (CMC). Administration of tranexamic acid and ABSE on mice tail wound shorten the bleeding time. In addition, the bleeding time of the ABSE group was 95 seconds, it means that it is shorter than that of the aspirin group. Therefore, the bleeding time of ABSE was similar to the bleeding time of the tranexamic acid group. It means that ABSE has a great efficacy as a hemostatic agent (Table 2).

Based on *one-way ANOVA* test results, a significance value of p = 0.005 (p < 0.01) was obtained. This means that there were significant differences in bleeding time. Furthermore, there was a marked difference between the CMC and aspirin groups (p = 0.004) (p < 0.01). Meanwhile, there were significant differences between the aspirin and tranexamic acid groups (p = 0.004) (p < 0.01) beside the significant differences between the aspirin and ambonese banana stem extract groups (p = 0.006) (p < 0.01). However, there was no significant difference between the tranexamic acid and

Table 2 The Mean and Standard Deviation of Bleeding Time

Groups	**Bleeding Time** $\overline{X} \pm SD$ **(Seconds)**
(K-) CMC	115 ± 2.5*
(K+) Aspirin	232.5 ± 8.7*
(P1) Tranexamic Acid	82.5 ± 1.7*
(P2) Ambonese Banana Stem Sap	95 ± 1.5

Note: *Indicated significantly different (p < 0.01).
Abbreviations: $\overline{X}$, mean; SD, standard deviation.

ambonese banana stem extract groups (p = 0.934). Aspirin has significantly shown prolonged bleeding time than CMC (p = 0.004), tranexamic acid (p = 0.004) and ABSE (p = 0.006); however, there was no difference between the tranexamic acid and ABSE group (p = 0.934).

Discussion

Ambonese banana stem sap contains tannins, flavonoids, and saponins as antibacterial components.[22] They also stimulate new cell growth in wounds. This sap can accelerate the re-epithelialization process of epidermal tissue, formation of new blood vessels (neocapilerization) and connective tissue (fibroblasts), and the infiltration of inflammatory cells in wounds. The sap-active substances that facilitate blood clotting are flavonoids, tannins and saponins.[26] Flavonoids and saponins play a vital role in inhibiting the cyclooxygenase cycle by decreasing the production of prostaglandins, which are responsible for vasodilation.[27] Tannins can also facilitate platelets adherence to sub-endothelial blood vessels, hence help release adenosine diphosphate (ADP) and thromboxane (TXA2) mediators.[28]

The active compounds in ambonese banana stem sap have a role in the process of hemostasis which is important to stop bleeding. There are many cells that play a role in this process such as platelets, which are activated by collagen exposure. Therefore, the glycoprotein on the cell surface adheres to the epithelium, which is mediated by von Willebrand factor (vWF). The adherence to the epithelial surface activates the membranes of alpha granules to release TXA2 and ADP which are considered major factors in platelet aggregation process. Hence, reducing the bleeding time of wounds or ruptured vessels. After aggregation, a coagulation cascade is initiated which is influenced by extrinsic and intrinsic factors.[11] Ambonese banana stem sap contains active compounds that are proven to accelerate wound healing. This is achieved by increasing PDGF-BB which is a growth factor released during platelet aggregation to activate the healing process. Therefore, when PDGF-BB production increases, it shows a high level of aggregation.

This research showed longer bleeding time in the aspirin group compared to the CMC, tranexamic acid and ambonese banana stem extract groups. The aspirin group was aimed to prolong the bleeding time. It was reported that administering aspirin for 7 days prolonged bleeding time.[29] This drug is a non-steroidal anti-inflammatory drug (NSAID) with blood-thinning property through the inhibition of thromboxane A2 (TXA2).[30] Therefore, it inhibits blood aggregation and causes prolonged bleeding time.[31] The platelet count in the negative control group was lower than that in the CMC, tranexamic acid and ambonese banana stem extract groups. Platelet count decreased in patients on aspirin medication, due to inhibition of aggregation, leading to prolonged bleeding time.

The tranexamic acid group had shorter bleeding time, followed by those given the Amboinese banana stem extract. These results are similar to the previous study which stated that tranexamic acid reduced bleeding time in patients who were given aspirin.[32] Meanwhile, there was platelets increase in the tranexamic acid group. This occurred because the compound is an anti-fibrinolytic drug that inhibits the breakdown of fibrin by plasmin through receptors found on plasminogen. These receptors prevent plasmin from binding and finally stabilize the fibrin matrix.[33]

Based on the results of the ambonese banana stem extract group, the bleeding time was shorter than the positive control group with an increase in platelet count. Furthermore, a test which stated that the use of 30% ambonese banana stem sap does not show any systemic toxicity on visible tissues in the fibroblast culture. However, it showed a large number of living fibroblasts accelerated wound healing through the activation of PDGF-BB.[19] The phytochemical test results also showed that the stem sap contained active compounds, including flavonoids, tannins and saponins, which have a therapeutic effect in the hemostasis process.

The low platelet count in peripheral blood smears compared to the tranexamic acid group is due to inadequate concentrations or dose, hence it affects the potency of the ambonese banana stem sap in hemostasis. The negative control group that was given CMC showed a normal hemostasis. Meanwhile, the positive control group that was given aspirin, tranexamic acid, and ambonese banana stem extract had a longer hemostatic process. The administration of tranexamic acid and ambonese banana stem extract led to a faster coagulation and increased platelets in the wound area, as shown through peripheral blood smears. Based on the discussion above, the use of ambonese banana stem extract in this study gave an effect similar to tranexamic acid as a hemostatic drug. Therefore, it can shorten the bleeding time through platelet activation.

Conclusion

The application of ambonese banana stem extract (*Musa Paradisiaca* var. *Sapientum* (L.) Kuntze) shortens the bleeding time in mice tail wound without interfering to platelet count. On the basis of the finding in this study, we have determined that it is essential to separate and purify the active chemicals that are found in plants. By utilizing the NMR technique, we can analyze the chemical structure in order to identify the active molecules that are involved in the process of blood coagulation. Certain blood coagulation parameters can be collected straight from the systemic circulation, which will result in a higher degree of certainty and a reduction in the amount of harm inflicted to animal models.

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Disclosure

The authors have declared that there is no conflict of interest.

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