The Effect of 96% Ethanol Extract of Bidara Leaf (Ziziphus Mauritiana) on the Hemostatic Activity in Sprague-Dawley Rats

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Abstract

Objective to investigate the efficacy of the 96% ethanol extract of Ziziphus mauritiana leaves on hemostasis activity in animal model study (Sprague-Dawley rats).

A laboratory experimental in vitro and in vivo study were conducted with Sprague-Dawley (SD) rats with randomized controlled design with a posttest-only control group design. Fifteen animals were used as research material, divided into 5 groups, negative control group (NCG), positive control group with golden standard hemostatic agent tranexamic acid 5% (TXA), 25% concentration of Z. mauritiana leaves extract (ZM25), 50% concentration (ZM50), and 100% concentration (ZM100). The bleeding time and the amount of bleeding were calculated by cutting the tip of the tails of the SD rats. PT, aPTT and TT were measured by taking blood samples from the SD rats. The data analysed using the Shapiro Wilk normality test, normally distributed data followed by a one-way ANOVA test with a significance level ($p<0.05$), continued with the Post-Hoc test.

The ZM100 group shows a significant difference with NCG, TXA and ZM25 groups in bleeding time. The NCG group shows a significant differences with all samples in the amount of bleeding. The ZM100 group shows a significant difference with all test groups in PT, aPTT and TT test.

The ethanol extract of Z. mauritiana leaves can accelerate bleeding time and the amount of bleeding, the extract can also stimulate the acceleration of PT, aPTT and TT factor in the bleeding cascade at high concentrations (100%).

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Introduction

Tooth extraction is one of the most frequently performed dental procedures, with bleeding as one of the most common complications.[1] Uncontrolled bleeding can threaten the patient's life and will interfere the dentists when performing tooth extraction procedure.[2] Incidence of bleeding after tooth extraction can be minimized by systemic or local interventions. Local post-extraction bleeding interventions can be carried out in various ways, some of which are by; pressure tampons, sutures, acrylic splints and the appliance of local hemostasis agents.[3]

In general, the wound healing process is divided into four phases, hemostasis, inflammation, proliferation, and remodelling phase.[4] Hemostasis occurs at the beginning of the wound healing process, followed by an inflammatory phase characterized by swelling and pain, then soft tissue structures re-form in the proliferation phase and end with complete tissue structure restoration in the remodelling phase.[4,5] Hemostasis (the process of bleeding to stop) is a complex mechanism influenced by interactions between the plasma coagulation cascade, fibrinolytic proteins, the vascular system, and thrombin (platelets).[6] The process of hemostasis is characterized by the occurrence of vasoconstriction and blood clot formation through activation of platelets and the coagulation cascade.[7]

Indonesia is a country that is rich in a variety of herbal plants that can be used as medicine. One of the plants in Indonesia that has many benefits is the Bidara plant or Indian Jujube plant (Ziziphus mauritiana). These plants are

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found in deserts, tropical and subtropical climates country.[8–10] Dried Z. mauritiana leaves are known to contain complex secondary metabolites such as tannins.[8] In several different tree samples, it was found that the tannin content in Z. mauritiana plants had the highest content in the leaves.[10] Ziziphus mauritiana is known to have various benefits such as antimicrobial, analgesic, antipyretic, anti-inflammatory and anticancer agent. Ziziphus mauritiana works pharmacodynamically and pharmacokinetically by increasing cellular activities, such as migration, proliferation, adhesion, and differentiation to improve wound healing.[11] In the phytochemical test and quantification Z. mauritiana has secondary metabolites such as alkaloids, flavonoids, saponins, tannins and terpenoids. Bark, fruit flesh and leaves of Z. mauritiana are known to have astringent properties so that they can be used to accelerate cessation of bleeding and accelerate wound healing process.[8,12] The astringent substances contained in some plants have a contraction effect, causing tissues to occur shrinkage.[13,14]

Tannin is an astringent substance that can precipitate thrombin which will then convert fibrinogen into a collection of fibrin fibers thread in the area of bleeding, these fibrin fibers will become a place for blood clotting proteins to attach so that the coagulation process can be achieved.[15] Tannins are also known to have a vasoconstrictor effect so that bleeding time will be shortened and the wound healing process can be achieved.[16,17]

As far as the researchers know, there is not much literature discussing the hemostasis activity of Z. mauritiana leaves. Based on the descriptions, researchers are interested in researching on the activity of 96% ethanol extract of Z. mauritiana leaves against the hemostasis process in experimental animal model study Sprague-Dawley (SD) rats.

Materials and methods

The type of study is laboratory experimental research in vitro and in vivo with randomized controlled design with posttest-only control group. This study was cleared for ethics by the Ethical Commission of the Faculty of Dentistry at Universitas Trisakti (No. 636/S2/KEPK/FKG/2/2023). The study procedures are the preparation of extract ethanol Z. mauritiana leaves, and phytochemical examination. The next procedures were in vivo and in vitro studies of the hemostasis activity of the 96% ethanol extract of Z. mauritiana leaves.

Preparation of Z. mauritiana Leaves Extract

Ziziphus mauritiana leaves weighing 4 kg were taken from the Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI) also known as Balittro, the leaves had been dried and identified by the National Research and Innovation Agency (BRIN). The dried powder leaves are macerated in the BioCore Laboratory, Faculty of Dentistry, Universitas Trisakti with 96% ethanol (Supelco-Merck, German) for 3 days and stirred every 4 hours before decanted and filtered. Then, the filtrate was evaporated with a rotary vacuum evaporator (Buchi R-215, Flawil, Switzerland) until a concentrated extract (crude) was obtained. To remove the remaining ethanol, the crude was heated at 45°C, the crude then dissolved with aquadest, and divided into several concentrations, 25%, 50%, and 100% Z. mauritiana extract ethanol.

Phytochemical Assay of Z. mauritiana Leaves

A phytochemical test was conducted at BioCore Laboratory to identify the active compounds contained in the 96% ethanol extract of Z. mauritiana leaves. The phytochemical compounds investigated in this study are flavonoid, steroid, terpenoid, alkaloid, and tannin.

To perceive the flavonoid composition, 5 mL dilute ammonia is added to a portion of an aqueous filtrate of the extract and then 1 mL concentrated sulphuric acid was added, the yellow colouration disappears when left to stand indicates the presence of flavonoids. To perceive steroid composition, 3 drops of concentrated hydrochloric acid and 1 drop of concentrated sulfuric acid are added to 2 mL of the extract, the colour changes to bluish green indicates the presence of steroids. To perceive terpenoid composition, 2 mL of chloroform added to the 1 mL extract and then added 3 mL of concentrated sulfuric acid, the colour changes to reddish brown indicates the presence of terpenoid. To perceive alkaloid composition, dragendorf reagent is added to the extract, the depositions of orangish sediment indicates the presence of alkaloid. To perceive terpenoid. To perceive tannin, 0.1% ferric chloride added to 1 mL sampel, the colour changes to bluish indicates

the presence of tannin.

In Vivo Assessments of Hemostasis

A total of 15 male SD rats were adapted for about 1 week for the acclimatization process, the need to eat and drink ad libitum. Experimental animals were randomly divided into 5 groups (n=3), negative control group (NCG) using 0.9% sodium chloride (NaCl) solution (B. Braun, Melsungen, Germany), positive control group with the golden standard drug tranexamic acid (TXA) solution (Bernofarm, Sidoarjo, Indonesia), treatment group with 25% Z. mauritiana leaves extract (ZM25), treatment group with 50% Z. mauritiana leaves extract (ZM50) and the treatment group with 100% Z. mauritiana leaves extract (ZM100).

The SD rats on each groups were anesthetized with ketamine 40 mg/kg BW (Agrovet Market, Lima, Peru) and xylazine 5 mg/kg BW (Interchemie, Venray, Netherlands). Later, the surgical area was cleaned using a 2% chlorhexidine solution and 1 cm distal segment from the tip of the tail of the rats were amputated with a scalpel blade no 15. Then, 0.1 mL of the solution was applied using an insulin syringe (Onemed, Sidoarjo, Indonesia). A digital chronometer is used to measure how long it takes to achieve hemostasis. Timing begins at the time of incision and stops when the bleeding at the site stops completely. To determine the amount of bleeding, each filtration paper (Whatman - Cytiva, Marlborough, US) was weighed in gram before the procedure with a precision laboratory scale (Sartorius M-Prove, Göttingen, Germany). After the bleeding was achieved, the filtration paper was used to absorb the bleeding that occurred in the wound on the SD rat's tail. This process is carried out until the bleeding stops as indicated by no more blood spots adhering to the filter paper. The difference in the weight of the filtration paper before and after the experiment was considered as the amount of bleeding.

In Vitro Assessments of Hemostasis

In the in vitro study, Prothrombin Time (PT), Activated Partial Thromboplastin Time (aPTT), and Thrombin Time (TT) are the hemostasis factors assessed in this study. Blood samples (n=5) were taken from SD rats in the previous in vivo study, blood sampling was carried out through the aorta and directly added to plastic tubes containing 0.2 mL sodium citrate 3.2% as an anticoagulant in a 1:9 (v/v) ratio to the blood. Blood samples were centrifuged at 3000 rpm for 10 minutes to separate blood plasma, then the supernatant plasma, which contains platelets, are divided in five sterile tubes for the coagulation test. The coagulation test such as PT, aPTT and TT were examined with reagents kit (Stago, Asnières, French) according to the manufacturer's instructions.

For the PT test, the plasma sample is divided into five tubes, each containing 0.1 mL of plasma which will then be treated by mixing the reagent with the research material. the first tube will only be given reagents as a negative control group, the second tube is given 0.1 mL 5% TXA as a positive control, the third tube is 0.1 mL ZM25, the fourth tube is 0.1 mL ZM50 and the fifth tube is 0.1 mL ZM100, all the tubes were incubated for two minutes at 37°C. Then, 0.2 mL of warmed PT reagent was added to the tube, and the clot formation time was calculated using digital chronometer from the start of the reagent drop until a coagulation formed at the bottom of the tube. The same protocols were delivered for aPTT and TT test depending on the reagents manufacturer's instructions, each protocol was repeated five times and assessed by blind investigators.

Statistical Analysis

The data were analysed using the Shapiro Wilk normality test, normally distributed data followed by a one-way ANOVA test with a significance level ($p \leq 0.05$), continued with the Post-Hoc test.

Results

From the results of the phytochemical test of the ethanol extract of Z. mauritiana leaves it was found that it contains active substances such as terpenoids, flavonoids, alkaloids, and tannins, while steroids are absence in this phytochemical test. The results of the phytochemical test are shown in Table 1.

The results of the bleeding time test shows that NCG group is significantly different ($p<0.05$) with all groups and the ZM100 group shows a significant difference ($p<0.05$) with the negative control, TXA and ZM25 groups. The results of the bleeding amount test shows the NCG group is significantly different ($p <0.05$) with all groups (Table 2). In the PT test results, the NCG and TXA groups show a significant difference ($p<0.05$) with the ZM50 and ZM100

groups. In the aPTT test results, the NCG group and the ZM100 group are significantly difference ($p<0.05$) with all groups. In the TT test results, the ZM50 group is significantly different with the TXA and ZM100 groups (Table 3).

Metabolit Sekunder	Metode	Hasil Uji
Terpenoid	2mL CHCl3 + 3mL H2SO4	+
Flavanoid	5mL NH3 + 1mL H2SO4	+
Steroid	3 drops of CH3COOH + 1 mL H2SO4	-
Alkaloid	Dragendorff's reagent	+
Tanin	1 mL FeCl 0.1%	+

Table 1. The phytochemical analysis of 96% ethanol extract of Ziziphus mauritiana leaves.

Treatment Group (n=3)	Bleeding Time (s)	Amount Bleeding (g)
NCG	4.11 ± 0.84 [a]	1.74 ± 0.15 [a]
TXA	2.40 ± 0. 15 [b]	1.33 ± 0.11 [b]
ZM25	2.08 ± 0.26 [bc]	1.27 ± 0.04 [b]
ZM50	1.50 ± 0.04 [cd]	1.36 ± 0.14 [b]
ZM100	0.94 ± 0.53 [d]	1.32 ± 0.17 [b]

Table 2. Hemostatic analysis of the bleeding time and amount of blood, for the different groups evaluated (in vivo).
Abbreviations: NCG, negative control (NaCl); TXA, positive control (5% Tranexamic Acid); ZM25, 25% concentration of Z. mauritiana leaves ethanol extract; ZM50, 50% concentration of Z. mauritiana leaves ethanol extract; ZM100, 100% concentration of Z. mauritiana leaves ethanol extract.
Note: The values represent the mean values and the standard deviation for the three animals evaluated in each group, different superscript letters (abcd) expressed statistically significant differences ($p<0.05$) in the lines.

Treatment Group (n=5)	PT (s)	aPTT (s)	TT (s)
NCG	37.58 ± 11.92 [a]	31.13 ± 0.89 [a]	14.64 ± 3.57 [abc]
TXA	36.43 ± 2.40 [ab]	20.14 ± 3.04 [b]	13.45 ± 2.57 [b]
ZM25	33.67 ± 4.99 [abc]	20.75 ± 1.39 [b]	18.78 ± 4.73 [c]
ZM50	26.21 ± 7.32 [c]	20.97 ± 2.69 [b]	21.53 ± 9.95 [ac]
ZM100	12.15 ± 1.90 [d]	17.10 ± 1.76 [c]	04.35 ± 2.43 [d]

Table 3. Hemostatic analysis of the PT, aPTT, and TT time, for the different groups evaluated (in vitro).
Abbreviations: NCG, negative control (Reagent); TXA, positive control (5% Tranexamic Acid); ZM25, 25% concentration of Z. mauritiana leaves ethanol extract; ZM50, 50% concentration of Z. mauritiana leaves ethanol extract; ZM100, 100% concentration of Z. mauritiana leaves ethanol extract.
Note: The values represent the mean values and the standard deviation for the three animals evaluated in each group, different superscript letters (abcd) expressed statistically significant differences ($p<0.05$) in the lines.

Discussion

Ziziphus mauritiana plant is believed to have benefits against various diseases. The potential health benefits of Z. mauritiana are known as anti-carcinogenicity, antioxidant, anti-inflammatory, immune stimulation, anti-obesity, gastrointestinal protective activity, hepatoprotective and inhibitor of foam cell formation in macrophages.[18] In this study, the leaves of Z. mauritiana is proven to contain several secondary metabolites such as terpenoids, flavonoids, alkaloids and tannins but not steroids. The findings of this study are in line with previous studies that reported Z. mauritiana leaves revealed the presence of alkaloids, terpenoids, phenolic substances, saponins, flavonoids, and tannins. The steroids in the studies are also absence.[19–21] In several different tree samples, it was found that the tannin content in Z. mauritiana plants had the highest content in the leaves.[10,20]

Tannins can accelerate the wound healing process through several cellular mechanisms, prevent free radicals and reactive oxygen species, promote wound vasoconstriction and increase the activity of forming capillaries and fibroblasts including the proliferation of keratinocytes.[22,23] Tannins have high hemostasis efficiency because they can conjugate with various proteins in the blood, causing instant coagulation.[24] Hemostasis is a process in which bleeding becomes reduced, and blood clots form to prevent further blood loss. Hemostasis occurs in two stages: the primary stage, in which the blood vessels contract causing a platelet plug to form, and the secondary stage, in which the plug is strengthened with fibrin strands to hold the clot in place through activation of the coagulation cascade.[25] Therefore, the astringent properties of tannins are expected to stop bleeding in damaged blood vessels, accelerate both the primary and secondary stages of hemostasis so the cessation of bleeding is completely achieved. Primary hemostasis can be assessed by bleeding time and amount of bleeding tests, while secondary hemostasis can be assessed by PT, aPTT and TT tests.[26]

In this study, the hemostasis activity of the ethanol extract of Z. mauritiana leaves was investigated using the rat tail tip amputation model for bleeding time and amount of bleeding test, which is a standard and reliable procedure for assessing the hemostasis properties of a new biomaterial.[25,27] The bleeding time test shows the negative control (NCG) had the longest bleeding time with 4.11 seconds and showed a significant difference ($p<0.05$) with all groups. While the ZM100 group was the group with the

fastest bleeding time with 0.94 seconds and showed a significant difference ($p<0.05$) with the NCG, TXA and ZM25 groups. The results of the bleeding amount test showed that the NCG group had the highest amount of bleeding with 1.74 grams, and showed a significant difference ($p <0.05$) with all groups.

These results are harmonious with studies investigating the therapeutic properties of tannins in Z. mauritiana. Tannins, expressively found in extracts, have demonstrated the ability to control bleeding through their astringent effects on contracting vessels and damaged tissues, accelerating blood proteins and supporting hemostasis.[21,25,26]

In the PT test shows, the negative control (reagent) and TXA groups show a significant difference ($p<0.05$) with the ZM50 and ZM100 groups. The ZM100 group is the group with the fastest PT with 12.15 seconds and shows a significant difference ($p<0.05$) with all groups. In the aPTT test shows, the reagent group was the group with the longest aPTT with 31.13 seconds, and shows a significant difference ($p<0.05$) with all groups. While the ZM100 group is the group with the fastest aPTT with 17.10 seconds and shows a significant difference ($p<0.05$) with all groups. In the TT test shows, the ZM50 group is the group with the longest TT with 21.53 seconds, and shows a significant difference with the TXA and ZM100 groups. While the ZM100 group was the group with the fastest TT with 4.35 seconds and shows a significant difference with all test groups.

This shows that the administration of ethanol extract of Z. mauritiana leaves can accelerate the process of secondary hemostasis at high concentrations (ZM100) compared to other control groups and lower concentrations. The higher the viscosity of the ethanol extract of Z. mauritiana leaves, the higher the composition of the active substance, so that wound healing can be improved due to its anti-inflammatory and antibacterial properties.[19]

Based on the results of the study, the ethanol extract of Z. mauritiana leaves showed therapeutic potential that could be used as a promising biomaterial for hemostasis agents in the clinical treatment of local bleeding. Further research is needed regarding the activity of the ethanol extract of Z. mauritiana leaves in a smaller concentration whether it can maintain the same efficacy and mechanism, and it is also necessary to carry out clinical trials in humans to confirm the hemostasis potential shown in this study.

Conclusions

The 96% ethanol extract of *Z. mauritiana* leaves can accelerate the cessation of bleeding time and the amount of bleeding, the extract can also stimulate the acceleration of extrinsic (PT) and intrinsic (aPTT) factors of the bleeding cascade, and accelerate the formation of fibrin clot (TT) at a high concentrations so that it can be developed as a topical hemostatic agent.

Declaration of Interest

The authors report no conflict of interest.

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