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Evaluation of the Antithrombotic Activity of *Acmella oleracea* L. Flower Ethanol Extract

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Abstract

Acmella oleracea L. (hereinafter abbreviated as AOE) is one of the plants with the potential for antithrombosis, one of the deadliest cardiovascular diseases in Indonesia. The antithrombotic activity test on AOE leaf extract revealed that it could lyse blood clots. However, no information regarding the AOE flower as an antithrombosis is provided. This study, thus, aims to determine the chromatography profile and the antithrombotic activity of the AOE flower ethanol extract. To identify the AOE chemical profile, thin-layer chromatography was carried out. Antithrombotic testing was performed on male rats of the *Sprague-Dawley* strain. Then, the antithrombotic activity was tested using the $FeCl_3$-induced rat method, with the observed parameter being total occlusion time. The test animals were also divided into six groups: normal, solvent (CMC-Na 0.9%), comparator drug (clopidogrel 8.67 mg/kg), and AOE (doses 125, 250, and 375 mg/kg). The data obtained were then analyzed statistically using *Kruskal-Wallis*, followed by *Tukey's*. The TLC profile results confirmed the presence of the alkaloid compound in AOE. The authors also found that AOE at doses of 125, 250, and 375 mg/kg significantly prolonged the occlusion time comparable to that of clopidogrel at 8.67 mg/kg ($p > 0.05$). This finding indicates that AOE has antithrombotic activity in $FeCl_3$-induced rats.

Keywords: *Acmella*; alkaloid; cardiovascular diseases; spilanthol; thrombosis; toothache plant

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INTRODUCTION

Thrombosis is a cardiovascular disorder that causes the biggest number of deaths in the world, including in Indonesia, with an increase in its prevalence of 1.5% each year.[1] In the hemostatic system, platelets are blood cells that play a role. If platelets are activated in the process of hemostasis, blood clots (coagulation), platelet aggregation, and thrombosis will be formed[2]. The presence of a thrombus formed from hemostatic disorders can cause blockages in blood vessels and

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trigger vascular disorders.[3] The antithrombotic disease is managed pharmacologically by employing frequently used modern antiplatelet anti-aggregation drugs, such as aspirin, clopidogrel, ticlopidine, and warfarin. Nevertheless, although several antithrombotic agents have been circulating on the market, their effectiveness and safety are still not optimal, so they have not been able to significantly reduce cardiovascular disease's prevalence. Specifically, Indonesia has many medicinal plants with various pharmacological activities, such as antioxidant, antimalarial, antiproliferative, anti-inflammatory, anticancer, antidiabetic, anticoagulant, antiplatelet, antithrombotic, and many more.[4–8] Therefore, developing and discovering new antithrombotic agents is still needed from various sources, including Indonesian medicinal plants.

One of the herbal plants widely known as an annual herb is *Acmella oleracea* (hereinafter abbreviated as AOE). The community empirically uses plants with reddish-green stems to treat toothaches. Phytochemical analysis of AOE reports that it consists of N-alkylamides, phytosterols, terpenoids, esters, and aromatic compounds.[9] These contents are found in all parts of the AOE plant and are most abundant in the flowers.[10] In this regard, one of the many N-alkylamide derivatives in AOE flowers is spilanthol. Pharmacological activities studied include analgesic, antioxidant, anti-inflammatory, diuretic, toothache medicine, and antimicrobial.[9] Several studies of AOE have tested its thrombolytic activity. Previous studies have uncovered that the AOE methanol and ethanol extracts have in vitro thrombolytic activity of 42.77% and 46.78%, respectively.[11] Based on a literature review, the ethyl acetate extract of AOE leaves has strong antioxidant activity with an IC_{50} value of 28.09 g/mL, which will be associated with a reduction in the effects of cancer and cardiovascular disease.[12] A previous study also showed that antioxidants have a positive impact on cardiovascular diseases.[13]

Nonetheless, research on the antithrombotic activity of ethanol extract from AOE flowers has not been reported. In fact, the use of an ethanol solvent for extraction has greater antithrombotic activity for blood clot lysis than other solvents.[11] Therefore, this antithrombotic activity was carried out to prove the thrombolytic activity of the ethanol extract of AOE flowers and identify the profiling of marker compounds in the extract. The antithrombotic activity could be determined by observing the occlusion time in animals infected with $FeCl_3$. This is a simple method of accessing antithrombotic activity and is sensitive to the presence of antiplatelet and anticoagulant activities in the tested samples.[14]

METHOD

Animals

The male Sprague-Dawley rats, eight weeks old and having a minimum body weight of 120 grams without physical defects, were used for this study. The number of rats used was 30, divided into six groups (each group consisted of five rats).

Extract

The ethanol extract of AOE was obtained from PT Konimex, Solo, Central Java, Indonesia. Certificate of analysis (CoA) results disclosed that the sample met specifications. To verify the validity of the plant raw material, the dried plant material was authenticated at the Department of Pharmaceutical Biology,

Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia (Voucher Number 08.5.10/UN1/FFA.2/S1/PT/2022).

Thin Layer Chromatography Analysis

The thin-layer chromatography analysis was done according to previous studies.[15,16] Shortly, AOE extract was dissolved in methanol and then spotted on a silica gel plate 60 F_{254} (Merck, Darmstadt, Germany). A mixture of n-hexane-ethyl acetate (2:1) has been optimized as the mobile phase.[17] After development, the plates were removed and dried. The chemical components were visualized under UV 254 nm and 366 nm and visible light after derivatization with the Dragendorff reagent. The present alkaloid is characterized by the presence of red-brown TLC spots.

Antithrombotic Activity

This study used a true experimental design. The research phase has obtained approval for the antithrombotic activity test protocol from the Research Ethics Commission of the Faculty of Medicine, Health, and Nursing (FK-KMK) at Universitas Gadjah Mada. The study was conducted at PT Konimex Laboratory, Sukoharjo, Central Java, to test antithrombotic activity. By giving $FeCl_3$ to test animals, the goal of this study was to find out if the ethanol extract of AOE flowers could prevent blood clots.

This study was divided into six groups consisting of normal, solvent (CMC-Na 0.9%), positive control (clopidogrel 8.67 mg/kg), and varying doses of the ethanol extract of AOE flowers, as in Table 1. The method of measuring the blood flow of rats induced by $FeCl_3$ refers to the previous method modified by [18], using a *Transonic Flowprobe Doppler Ultrasound* tool. Rats acclimatized for seven days were given the treatment, as displayed in Table 1, before the induction of $FeCl_3$.

Table 1. Distribution of test animal groups

Group	Carrier	Induction of $FeCl_3$
Normal	Water	No
Solvent	CMC Na 0.9%	Yes
Clopidogrel 8.67 mg/kg	CMC Na 0.9%	Yes
AOE 125 mg/kg	CMC Na 0.9%	Yes
AOE 250 mg/kg	CMC Na 0.9%	Yes
AOE 375 mg/kg	CMC Na 0.9%	Yes

(AOE: *Acmella oleracea* L. flowers ethanol extract)

The test preparations were administered orally for eight days, once daily. The dissection of the test animals was carried out three hours after the administration of the last test preparation, which was on the eighth day. The test animals were anesthetized with 1 ml of sevoflurane by inhalation. After the test animals fainted, they were given subcutaneous injections using 10 mg/kg ketamine. Rats were then supined on a surgical board for shearing and surgery from the mandible to the suprasternal notch. Bleeding reduction was made by dripping 0.1 ml of hemoblock, then letting stand for 1-2 minutes and cleaning with cotton soaked in 70% alcohol. Then, the rats' blood flow was measured with *Transonic Flowprobe Doppler Ultrasound* on the right carotid artery side (every 15 seconds for five minutes). After measuring, the blood vessels were cleaned, followed by administration of 5% $FeCl_3$ by placing a piece of Whatman paper (1 x 0.5 cm) that

had been moistened on the surface of the carotid artery (for three minutes) (Surin, 2010). After exposure, the paper was removed, and the vein was cleaned with a saline solution. The induced area was measured for a decrease in blood flow over time, every 15 seconds for 40 minutes.[19,20]

Statistical Analysis

Data on occlusion time measurements were obtained and analyzed using Statistical Product and Service Solution (SPSS) v.26 (IBM) software with a 95% confidence level. First, it was tested for normality with the Shapiro-Wilk test to determine whether the data were normally distributed, then tested for homogeneity with the Levene test. Furthermore, a different test was carried out using the Kruskal-Wallis test to find out whether there was a significant difference between the sample groups and continued with Tukey's test to determine more specifically the location of the differences between the sample groups.

RESULTS AND DISCUSSION

Thin Layer Chromatography Analysis

AOE extract was identified for its phytochemical profile using thin-layer chromatography (TLC). The identification aimed to determine the marker compounds contained in the extract. The amount of extract spotted was 20 µl on the plate. The elution results were also observed with 254 nm and 366 nm UV light. TLC results can be seen in Figure 1. The TLC results reveal that AOE had one dominant spot in Figure 1C with Rf 0.28. This spot appeared red-brown after being sprayed with Dragendorff reagent (Figure 1D). Dragendorff reagent was used to detect the presence of alkaloids, especially with the secondary and tertiary amine groups. It indicates that AOE contained alkaloids as the main compound.

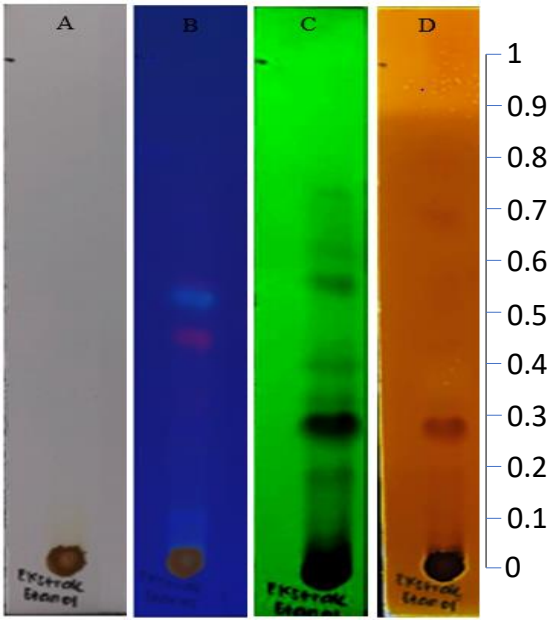

Figure 1. TLC profile of AOE extract. The stationary phase was silica gel F_{254} with an elution distance of 8 cm. The mobile phase was N-Hexane: ethyl acetate (2:1). The detection was carried out: (A) Visible light; (B) UV 366 nm; (C) UV 254 nm; and (D) Dragendroff spray reaction. The major spot at the Rf of 0.28 indicates the presence of an alkaloid.

Antithrombotic

To investigate the antithrombotic activity of the ethanol extract of AOE flowers, $FeCl_3$-induced thrombus formation in rats was performed. The AOE dose series used in this study were 125, 250, and 375 mg/kg. To find out the differences between each dose variation in the normal treatment group, solvent, and comparator drug, statistical analysis was done using one-way ANOVA with a 95% confidence level with SPSS.

The statistical analysis results of total occlusion time data exposed that the positive control (clopidogrel 8.67 mg/kg) and AOE with all doses used could significantly protect test animals against thrombus formation compared to the solvent group ($p<0.05$). The statistical analysis results also showed no significant

difference between the positive control (clopidogrel 8.67 mg/kg) and AOE doses of 125, 250, and 375 mg/kg ($p > 0.05$). As such, it can be concluded that AOE at doses of 125, 250, and 375 mg/kg could inhibit thrombus formation equivalent to clopidogrel 8.67 mg/kg as the comparator drug. In addition, the average total occlusion time for AOE doses of 125, 250, and 375 mg/kg was 29, 35, and 28 minutes, respectively, and for clopidogrel doses of 8.67 mg/kg, it was 30 minutes. The graph of total occlusion time in the test animals can be observed in Figure 2. In this case, the formation of a thrombus in the blood vessels will affect the blood flow velocity in the test animals. It is characterized by a decrease in blood flow velocity of up to 0.0 mL/minute on the *Transonic Flowprobe Doppler Ultrasound* screen, and total occlusion time is obtained.[14] This method can be used for antithrombotic testing, as evidenced by the workings of solvent groups and comparator drugs. These results provide scientific evidence regarding AOE antithrombotic activity.

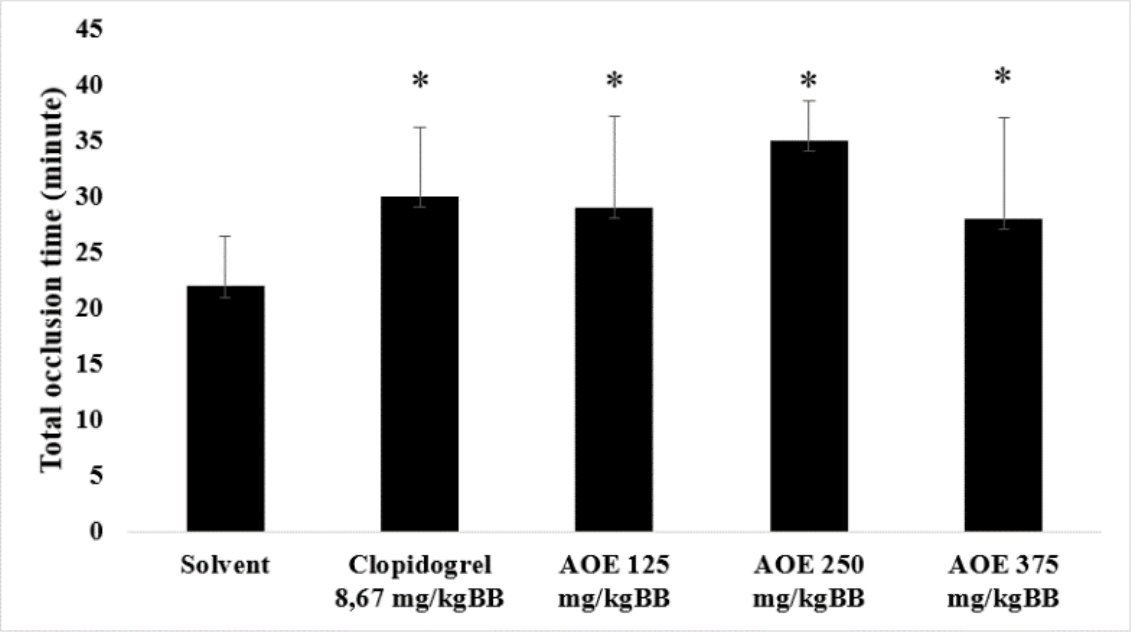

Figure 2. Graph of total occlusion time in test animals using $FeCl_3$-induced *Sprague-Dawley* rats. (*p<0.05, significant to solvent)

Further, free radicals generated by $FeCl_3$ will cause lipid peroxidation, which ends in damage to endothelial cells. This vascular surface damage can lead to platelet activation. The first stage of platelet activation begins with the adhesion of platelets to the vessel wall. Platelet adhesion involves collagen receptors, i.e., GPVI. This platelet adhesion will also lead to platelet activation, recruitment, and aggregation. In addition, CYP metabolizes the comparator drug clopidogrel in the liver and binds irreversibly to the platelet P_2Y_{12} receptor. Aggregation occurs when ADP binds to the P_2Y_{12} receptor, resulting in a change in shape, causing a signal sequence, and forming platelet aggregation and thrombus stabilization. In this study, the authors found that AOE could prolong the occlusion time in thrombotic rats induced by $FeCl_3$. It indicates that the extract has the potential to be developed as an antithrombotic agent by targeting antiplatelet and anticoagulant mechanisms, as this method is sensitive to both thrombosis-

related mechanisms.[14,21] Previous studies have indicated that AOE has strong antioxidant activity with an IC_{50} of 28.09 g/µL (DPPH method). As antioxidants have an association with the prevention of cardiovascular diseases, the antioxidant compounds in AOE might contribute to their antithrombotic activity.[12,13] Moreover, this finding demonstrates that AOE has the potential to be further developed as an antithrombotic agent. Several other plants, such as *Allium sativum, Rosmarinus officinalis, Boswellia serrata, Sesamum indicum, Matricaria chamomilla,* and *Carthamus tinctorius,* have also been previously published for their antithrombotic activities.[22] This finding corroborates previous studies, providing additional data regarding the potency of Indonesian medicinal plants as a drug candidate.[23–26]

CONCLUSION

At doses of 125, 250, and 375 mg/kg, the ethanol extract of AOE flowers has antithrombotic activity because it stops rats from getting thrombi when $FeCl_3$ is given. The extract also contains alkaloids as a major compound. Hence, further investigation is needed to identify the active compound.

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