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ORIGINAL RESEARCH

Antithrombotic potential of ethanol extract from *Gardenia jasminoides* leaves planted in Aceh

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Abstract. Therapeutic agents from plants are believed to be effective, sustainable and readily available which stimulates researchers to explore various activities of plants. This study aimed to investigate the activity of *Gardenia jasminoides* (*G. jasminoides*) leaves in enhancing the length of bleeding time and clotting time thus can be optimized to be a potential antithrombotic agent in the future. The animals were classified into 5 groups included 3 treatment groups receiving *G. jasminoides* leaves (GJL) extract at dose 100, 200, 300 mg/kg Body Weight (BW), 1 positive control receiving aspirin 0.208 mg/20 g and 1 negative control. The result showed that the administration of GJL extract at dose 100, 200 and 300 mg/Kg BB and aspirin prolong the bleeding time and clotting time. The highest dose showed effects as strong as positive control. Overall, the effect of GJL 300 mg as antithrombotic agent is as potent as Aspirin.

Keywords: *G. Jasminoides* leaves, bleeding time, clotting time

INTRODUCTION

The hemostasis under normal circumstances helps to stop bleeding and control the response to the excessive bleeding. Disturbances in hemostasis caused by the presence of endothelial dysfunction or hypercholesterolemia potentially leads to the formation of thrombus. The accumulated thrombus and cholesterol in the blood that settled in the arteries blood vessels causing atherosclerosis. Medications used to address this problem that work by inhibiting clot formation are known as antithrombotic agents [1].

Antithrombotic agents such as aspirin and clopidogrel are often prescribed to improve blood circulation and often referred as blood-thinner medication. Some studies showed an increase in the number of patients with inadequate response to aspirin. In addition, a study reported the failure therapy with aspirin in which patients who took the medication in a longer time, results in the suboptimal therapy outcome [2]. Another study also found that more than 75% of ischemic stroke patients treated with 80 mg aspirin did not produce the targeted platelet aggregation response [3]. Aspirin also often cause side effects such as dyspepsia and gastric bleeding [4]. The failure of aspirin in inhibiting platelet aggregation could exacerbate patient condition thus elevating the risk of patients having recurrent stroke, heart attack or even death. This failure should urge researcher to discover new drugs with less adverse effects in addressing the problem of blood clotting.

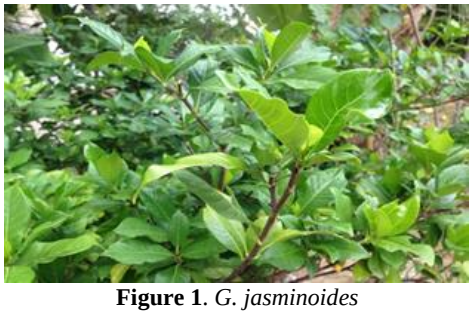

Figure 1. *G. jasminoides*

Research on antithrombotic effects has been examined in several plants, one of which is on *G. jasminoides* (Figure 1) by Zhang (2013) who examined the dried fruit extract of *G. jasminoides* in Karagenan-induced rats. The

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water extracts and the isolated compound, iridoid glycosides from dried fruit showed an antithrombotic effect [5]. To date, several studies had been conducted on *G. jasminoides* fruits. However, the evaluation on the antithrombotic activities of the leaves, especially those which are harvested in Aceh is limited.

METHODOLOGY

Animals

Mice (*Mus muculus*) were obtained from the Faculty of Veterinary Medicine Universitas Syiah Kuala

Materials

Leaves *G. jasminoides* was collected from Langsa Baro District, Langsa City, Aceh. Leaves were determinated at the Herbarium Bogoriense Center for Biology Research LIPI, Bogor, West Java.

Preparation of *G. jasminoides* leaf extract

G. jasminoides leaves (7 Kg) were washed, cleaned and drained. The leaves were scraped with flannel and dried under sunlight (4 weeks). Dried leaves (figure 2) were then grounded to yield leaves powder. The powder (800 gram) was soaked with 7.5 parts of ethanol 70% of 6 L and allowed for 5 days while stirred occasionally. Then mixture was then filtered. The residue was then soaked 1 with 2.5 parts of ethanol 70% (2 L) for 2 days. Repeated procedure was applied to second mixture. All the filtrate was mixed. The final filtrate was evaporated using a rotary evaporator (50^{o}C) to yield a viscous liquid [6].

Figure 2. Dried leaves of *G. jasminoides*

Test the antithrombotic effect of the leaf ethanol extract *G. Jasminoides*

The mice were adapted for one-week acclimatization in order to adapt to the laboratory atmosphere. The mice were divided into 5 groups and each group consisted of 5 mice. Mice were fed and drunk *ad libitum*. The bleeding and blood clotting time (day 0) were recorded before treatments were given. The animals were divided into 5 groups as follow:

A	:	Positive control group received suspension of aspirin 0.208 mg/20 g BW
U	:	untreated group, received water *ad libitum*
GJL-100	:	received suspension of leaf ethanol extract *G. jasminoides* 100 mg/Kg BW
GJL-200	:	received suspensions of leaf ethanol extract *G. Jasminoides* 200 mg/Kg BB
GJL-300	:	received suspension of a leaf ethanol extract *G. Jasminoides* 300 mg/Kg BB

The animals were given extract or aspirin orally for one month. The bleeding and blood clotting time were recorded on the 7th, 14th, 21st, and 28th day.

The calculation of blood clotting time is started when the blood is dripped on the preparation until fibrin threads are formed and stated in seconds [7]. The bleeding time is measured by the time blood comes out of the tail of the injured mouse and is absorbed by filter paper until the blood stops being absorbed by filter paper and is expressed in seconds [8,9].

Phytochemistry analysis

Glycosides

GJ extract (500 mg) was dissolved in 1 mL ethanol. The mixture was then evaporated on water bath. The resulted residue was dissolved in 5 mL of the concentrated anhydrous acetic acid and added 10 drops of concentrated H_2SO_4. The presence of glycoside compounds is confirmed by the change of the mixture into blue or green [10].

Flavonoid

A total of 500 mg of ethanol extract of *G. Jasminoides* was added with 10 mL of aquadest, 5 mL of dilute ammonia and 1 mL of concentrated H_2SO_4. The presence of flavonoids is characterized by the formation of yellow in the reaction tube [12].

Saponins

A total of 50 mg of ethanol extract of *G. Jasminoides* was added with 10 mL of hot water in the reaction tube, it was then shaken vigorously for 10 minutes. Positive saponins are characterized by the formation of two layers or

froth in the reaction tube as high as 1-10 cm which was not disappear with the addition of HCl 2N [10].

Tannins
A total of 500 mg of the leaf ethanol extract *G. Jasminoides* was diluted with the aquadest to form a colorless mixture. Then it was added with a few drops FeCl3 5% and observed for color changes occurring. Positive tannins are characterized by the formation of green or blue in the reaction tubes [10].

Steroids
A GJ extract was added with 2 mL of chloroform, and concentrated H_2SO_4. The presence of steroids is characterized by the formation of red/brownish mixture on the chloroform layer [11].

Terpenoids
GJ extract (800 mg) was added with 10 mL of methanol, shaken slowly then filtered. The filtrate was added with Chloroform (2 mL) and diluted H_2SO_4 (3 mL). The presence of terpenoids is characterized by the formation of reddish color in the reaction tube [12].

Bleeding time (bleeding time)
The timing of bleeding is conducted using the modified Duke method. The tail squeaky inserted into the holder then cleaned the tail of the mice with alcohol 70%. The tail mice were wounded using blood lancet. Blood that drips from a squeaky tail is absorbed using a filter paper. Replace filtered paper every 30 seconds. The time Interval when blood is first drip until blood stops dripping is called bleeding time. Normal bleeding time using this method is 1-3 minutes [8,9].

Blood clotting time (clotting time)
The timing of blood clotting is conducted by a drop method, where blood samples are taken through the mice that are injured using blood lancet. The blood coming from the tail of the mice was shed on the glass of clean and dry preparations. The blood is then removed using a needle every 30 seconds until the presence of fibrin yarn. Blood clotting time is recorded when blood is sprinkled over the preparation until the formation of fibrin yarn. The normal time for blood clotting using the Drip method is 2-4 minutes [7].

Data Analysis
Statistical analysis was carried out using one-way ANOVA (analysis of variance). The difference between the treatment groups for each recording time (7th, 14th, 21st, and 28th day) was identified using LSD (Least Significant Differences).

Ethical clearance

The study has been approved by ethic committee of Dentistry Faculty, Universitas Syiah Kuala

RESULTS AND DISCUSSION

The mechanism involved in haemostasis including adhesion, activation and aggregation of platelets which produce platelet plugs [13]. The interfering in those steps resulted in the plugs obstruction shown by the length bleeding time and inclined clot formation time. Antithrombotic effect of drugs or herbal was investigated in two methods including evaluation of bleeding time and blood clotting time. Bleeding time indicates the time required by platelet to close the wound, while blood clotting time indicates the required time for coagulation mechanism that occurs to produce fibrin and strengthens the wound closure by platelet [13]. The observation was conducted for 28 days. The bleeding time and blood clotting was recorded on day 0, 7, 14, 21 and 28. The observation time range is taken based on the lifetime of platelet which is 7 to 11 days. The positive group was given aspirin due to its mechanism of action. Aspirin inhibits the cyclooxygenase enzyme which then slows down the formation of thromboxane A_2, one of the essential components in blood.

The activity of *G. jasminoides* in increasing bleeding time

The effect of GJL extract is shown in Table 1. The administration *G. jasminoides* extract and aspirin lengthen the bleeding time

Table 1. Bleeding time

Groups	Day 0	Day 7	Day 14	Day 21	Day 28
A	61.4	113.8^{a}	190.1^{a}	204.4^{b}	247.5^{b}
U	59.1	55.8^{d}	55.5^{d}	56.2^{e}	55.9^{e}
GJL-100	59.1	69.4^{c}	74.9^{c}	101.6^{c}	173.6^{d}
GJL-200	56.4	93.0^{b}	125.3^{b}	156.8^{d}	217.3^{c}
GJL-300	58.2	112.3^{a}	186.8^{a}	216.2^{a}	290.5^{a}

The different letters at values are significantly different.

The increased bleeding time started on the day 7 to day 28. There is no increase in the untreated group. The increased dosage of extracts prolongs the bleeding time. The extract at dose 300 mg/Kg BB showed longer bleeding time compared to the extract dose 100 and 200

mg/Kg BB. Statistical analysis shows that the bleeding time of aspirin and GJL extracts differ significantly against negative control. These results indicate that the extract at dose 100, 200 and 300 mg/Kg BB increase the blood clotting time ($P < 0.05$) as good as aspirin does. There is significant difference among all groups on day 7 and 14. On day 21 and 28 bleeding time of GJL-300 increased continuously so that it differs significantly against positive control. The GJL-300 shows a greater effect in increasing bleeding time compared to the positive control (P = 0,000).

The activity of *G. Jasminoides* in increasing clot formation time

The increased blood clotting time observed in aspirin and GJL extracts groups during 28 days (Table 2). The statistical analysis shows significant difference between aspirin and GJL extracts to untreated group ($P < 0.05$). No significant difference between GJL-300 and aspirin groups analyzed by LSD (P = 0,068).

Table 2. Blood clotting time

Groups	Day 0	Day 7	Day 14	Day 21	Day 28
A	116.3	133.4^{a}	176.5^{a}	204.2^{a}	245.3^{ab}
U	115.5	118.0^{b}	118.8^{c}	124.9^{b}	117.7^{c}
GJL-100	114.4	129.9^{a}	137.8^{c}	162.5^{a}	186.8^{b}
GJL-200	116.2	133^{a}	142.9^{bc}	165.9^{a}	199.1^{b}
GJL-300	114.3	135^{a}	160.8^{b}	194^{a}	256^{a}

The different letters at values are significantly different.

This result shows that GJL displays an antithrombotic effect. An antithrombotic agent helps to prevent the capillary obstruction caused by clot formation as a risk factor for heart and capillary diseases. The therapeutic activity shown by herbal is possibly due to the presence of secondary metabolite compounds such as glycoside terpenoids, flavonoids and saponins. Iridoid, classified into glycoside terpenoid, was reported to inhibit phospholixase-A_2, an enzyme that play role in the formation of arachidonic acid [14]. Arachidonic acid increases the activation of platelet, induces vasoconstriction and stimulates platelet aggregation. Flavonoids inhibit the aggregation of platelets by inhibiting cyclooxygenase [15]. Saponins blocks vasoconstrictor effect of calcium [14]. Because of the role of these three compounds so that the blockage formation process can be inhibited.

Phytochemical analysis shown in Table 3 on GJL extract confirmed the presence of terpenoids glycosides, flavonoids and saponins. A study conducted by Zhang (2013) water extracts of dried fruits of *G. Jasminoides* and its isolates showed the antithrombotic effects observed as the blockage of vein thrombus in mice.

Table 3. Phytochemistry test

Phytochemical compounds	Result
Alkaloid	+
Flavonoid	+
Saponin	+
Tanin	+
Steroid	+
Terpenoid	+
Glicoside	+

The larger effect does not guarantee the safety of an active substance so that the testing to determine the safety of an active substance before it is developed into medicinal material becomes the basis in the development of new medicines. It is therefore very important to conduct acute toxicity testing (LD-50) for the ethanol extract *G. Jasminoides* as an Antithrombotic agent.

CONCLUSION

To conclude, ethanol extract of leaves of *G. jasminoides* doses of 100, 200 and 300 mg/kg BW showed antithrombotic effects by increasing bleeding time and blood clotting time. The highest dose (300 mg/kg BW) displayed a greater effect in increasing bleeding time compared to positive controls (p = 0,000) and comparable effect in increasing clotting time to positive control (p = 0.068).

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