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Antithrombotic and antihemolytic effects of *Lagerstroemia speciosa* (Lythraceae) aqueous extract

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ABSTRACT

Introduction: Aqueous extract of *Lagerstroemia speciosa* (EALS) (Lythraceae) is widely used to treat diabetes. This plant has been shown an *in vitro* thrombolytic activity that indicates its potential to prevent the formation of blood clots *in vivo*. Thus, this study was undertaken to evaluate the antithrombotic and antihemolytic effects of EALS.
Methods: Rats of both sexes (200 ± 5 g) were divided into five groups of six animals. Each group received orally distilled water, EALS (250, 500, 1000 mg/kg), and acetylsalicylic acid (100 mg/kg) for five days. After treatment, the FeCl3-induced arterial thrombus formation method was used to determine occlusion time. A coagulometer was used to detect activated partial thromboplastin time (aPTT) and prothrombin time (PT). Rabbit blood was used to determine clot lysis activity *in vitro* and antihemolytic activity using the 2,2-azobis hydrochloride (2-methylpropionamidine) (AAPH) method.
Results: EALS increased the occlusion time in a dose-dependent manner. At the dose of 1000 mg/kg, EALS increased the occlusion time significantly, from 4.59 ± 2.45 minutes to 15.52 ± 2.38 minutes (P < 0.01). At high concentrations (1-4 mg/mL), EALS showed a significant increase in aPPT and PT (P < 0.05). Streptokinase and EALS (4 mg/mL) induced significant clot lysis with percentage values of 78.48 ± 2.2 % and 49.5 ± 1.53 %, respectively (P < 0.001). EALS inhibited AAPH-induced hemolysis.
Conclusion: EALS exhibited antithrombotic and antihemolytic activities. The antithrombotic property of the plant could be attributed to its anticoagulant and thrombolytic activities. Regular consumption of *L. speciosa* leaves may prevent or treat thrombotic diseases.

Implication for health policy/practice/research/medical education:
The incidence of thrombotic complications is increasingly recognized as a major factor in some diseases like COVID-19. This research provides a safe and natural product such as *Lagerstroemia speciosa* leaves for prevention and treatment of thrombotic disorders.
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Introduction

In Sub-Saharan Africa, cardiovascular diseases are the major causes of death, responsible for approximately 13% of all deaths and 37% of all non-communicable disease death (1). Thrombosis is one of the leading causes of thromboembolic disorders affecting millions of persons worldwide (2). An insoluble blood clot becomes a thrombus and may lead to acute myocardial infarction, arterial embolism, and ischemic stroke (3).

Since the discovery of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19), millions of cases have been diagnosed worldwide, resulting in hundreds of thousands of deaths. Recent clinical data have highlighted that COVID-19 is associated with a significant risk of thrombotic complications ranging from microvascular thrombosis, venous thromboembolic disease, and stroke (4). The impact of thrombotic disorders on modern

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societies is enormous because of the loss of lives, the loss of quality of life of surviving patients, and its huge socioeconomic burden (5).

The use of antithrombotic agents, including anticoagulants, antiplatelets and thrombolytics, has been increased in the past decade and is expected to continue to increase (6). However, these drugs have certain limitations associated with their clinical applications, such as bleeding complications (7). Recently, several investigations into a new antithrombotic agent have been documented. Plants can serve as alternative sources for developing new medicines due to their biological activities. (8,9).

Lagerstroemia speciosa is a medicinal plant from the family Lythraceae, widely used for treating diabetes (10). Pharmacological studies have shown that the leaves of this plant possess thrombolytic (11) and diuretic (12) activities. The thrombolytic property of *L. speciosa* is believed to reveal its likely ability to prevent the formation of blood clots *in vivo*. The purpose of this work is to study the antithrombotic effects of *L. speciosa* in rats, in order to develop a potential remedy for the prevention or treatment of thrombotic diseases.

Materials and Methods

Plant material and extract preparation

Fresh leaves of *L. speciosa* were collected in Cocody (Cote d'Ivoire), at coordinates (5°21'N3°58'W), in July 2021. The botanical identification of the material was performed by Doctor Konan Yao and a voucher specimen was deposited at the Herbarium from the National Floristic Center of Felix Houphouet Boigny University (UCJ 01 17 44). The decoction of fresh leaves was used for extract preparation. Nine hundred and fifty grams (950 g) of fresh leaves of *L. speciosa* (Lythraceae), devoid of petioles, were washed with distilled water. The leaves were brought to a boil in 8.5 liters of distilled water for 30 minutes. After cooling, the decoction was filtered 4 times (two filtrations on white cloth and on cotton wool). The filtrate obtained was placed in an oven at 55°C. After water evaporation, a dry extract of *L. speciosa* was obtained and stored at 5°C.

Animals and ethics

A variety of animals were utilized in our study. Rats (*Ratus norvegicus*) and rabbits (*Oryctolagus cuniculus*) weighing 215 ± 5 g and 1.8 ± 0.4 kg, respectively, were obtained from the Animal House of the Laboratory of Biology and Health of UFR Biosciences at Cocody University in Abidjan (Côte d'Ivoire). They were housed in a constant temperature room with a light/dark cycle of 14/10 hours. All animals were fed and given water *ad libitum* until use.

Experimental procedures and protocols used in this study were approved by the ethical committee of Health Sciences, University Felix Houphouet-Boigny of Cocody-Abidjan. These guidelines were in accordance with the internationally accepted principles for laboratory use and care (13).

Drugs and reagents

2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) was purchased from Sigma-Aldrich (USA). Prothrombin, cephalin-kaolin, and Calcium chloride ($CaCl_2$ 0.025 M) were obtained from Cypress Diagnostics (Belgium). Ferric chloride, ascorbic acid, and streptokinase (SK) were purchased from Merck (Germany). All other drugs and reagents used were of analytical grade.

Phytochemical screening

Phytochemical screening was performed to highlight the major chemical groups, such as alkaloids, saponins, flavonoids, polyphenols, tannins, quinones, sterols, and polyterpenes, using standard procedures (14,15).

Blood collection and red blood cell preparation

Six rabbits were anesthetized with ketamine (100 mg/kg) and the blood was collected from the saphena vein. Erythrocytes were isolated and stored according to the method described previously (16) with slight modifications. The blood samples collected were centrifuged at 3000 rpm for 10 minutes. Red blood cells were separated from the plasma and buffy coat and washed three times by centrifugation (3000 rpm, 5 minutes). The supernatant and buffy coats of white cells were carefully removed with each wash. The washed erythrocytes were stored at 4°C and used within 6 hours for further studies.

Hemolytic test

The hemolytic activity of the *L. speciosa* extract was done by a previously described method (17). Two concentrations of *L. speciosa* extract (10^{-3} and 10^{-1} mg/mL) were added to 20% red blood cell solution. The saline solution NaCl (0.9%) (positive control) and distilled water (negative control) were also added separately to the 20% red blood cell solution. The mixture of 0.2 mL of 20% red blood cell solution and 0.8 mL of L. speciosa was incubated for 30 minutes at 37°C and then centrifuged at 3000 rpm for 10 minutes. The percentage of hemolysis was determined at the longwave of 470 nm and expressed as:

*% Hemolysis = [AE / AC] * 100*, with AE: Absorbance of the sample and AC: Absorbance of the control (hypotonic solution).

Antihemolytic test

The *in vitro* study of the antihemolytic effect of the extract of *L. speciosa* was carried out according to the AAPH method (2, 2-azobis 2 amidino-propane-dihydrochloride) (18). This assay highlighted the protective effect of *L. speciosa* on red blood cell membranes. The standard control used for the study was ascorbic acid. Conveniently, it was added to 200 µL extracts or ascorbic acid at different concentrations (10^{-1} -1 mg/mL), 200 µL of 20% red blood cells. The mixture was incubated for 30 minutes at 37°C. 400 µL of AAPH (200 mM) was added to the mixture and incubated again at 37°C for 2 hours. Before centrifugation of the mixture at 1200 rpm for 10 minutes, 3 mL of PBS was added. The percentage of inhibition of hemolysis was determined at the longwave of 540 nm, as follows:

% Inhibition of hemolysis = [1- (AE / AC)] * 100, with AC: Absorbance of the positive control and AE: Absorbance of the sample.

Effects of *Lagerstroemia speciosa* on blood coagulation

Preparation of platelet-poor plasma

Platelet-poor plasma preparation was realized according the method described by the professional order of medical technologists of Quebec (19). Platelet-poor plasma was separated from citrated rabbit whole blood. The whole blood was centrifuged at 2500 rpm for 15 minutes. The supernatant obtained, was removed without disturbing the pellet. To be sure that the plasma was devoid of platelet, second centrifugation was operated at 2500 rpm for 10 minutes. The new plasma was taken without cellular debris and stored at –20°C until used.

Activated partial thromboplastin time (aPPT) assay

Intrinsic and extrinsic pathways of coagulation were determined according to a previously described method (20), with slight modifications. Plasma (43 µL) was pipetted into clotting tubes and warmed for 2–3 minutes at 37°C. Then, 7 µL of distilled water (for control), and of plant extracts (10-1- 1 mg/mL) for the test were added. Cephalin-kaolin reagent (50 µL) and calcium chloride (50 µL) were added to the mixture, respectively. Cephalin-kaolin reagent and calcium chloride were pre-warmed at 37°C for 2-3 minutes. The coagulation time was recorded with a coagulometer (CyanCoag, Belgium).

Prothrombin Time (PT) assay

To explore the extrinsic pathway of coagulation, 43 µL of plasma was pipetted into clotting tubes and incubated for 2–3 minutes at 37 °C. Then, 7 µL of distilled water (for control), and of plant extracts (10^{-1}- 1 mg/mL) for the test, were added to clotting tubes. Prothrombin reagent (100 µL), pre-warmed at 37°C for 2-3 minutes, was added to the mixture. The coagulation time was recorded with a coagulometer (CyanCoag, Belgium).

Thrombolytic activity

Streptokinase (SK) preparation

Streptokinase (1 500 000 I.U.) was used as a standard. Sterile distilled water (5 mL) was added to streptokinase vial and mixed properly. From this suspension 100 µL (30 000 I.U.) was used for *in vitro* thrombolysis (21).

Clot lysis assay

Clot lysis studies were carried out as reported earlier (21). Rabbit's blood (10 mL) was distributed in preweighed sterile centrifuge tubes (0.5 mL/tubes) and incubated at 37°C for 45 minutes. After clot formation, the supernatant was completely removed without disturbing the clot. Each tube containing clot was weighed again to calculate the clot weight. 100 µL of EALS (5 mg /mL), 100 µL of streptokinase and 100 µL of distilled water were added separately in different tubes containing clots. Streptokinase and distilled water were used as positive and negative controls, respectively. The mixtures were incubated for 90 minutes. Released fluid was removed and the tubes were again weighed to determine the difference in weight after clot disruption. The experiment was repeated (n=4). The percentage of clot decrease was calculated using the following equation:

% DC = [(M1-M2)] / M1 × 100, with % DC: percentage of clot decrease, M1: initial weight of the clot, M2: final weight of the clot.

Acute oral toxicity

The acute oral toxicity of *L. speciosa* extract was performed according to the OECD 423 guidelines (22). Nine female rats were divided into three groups of three animals. The first group received distilled water used as a reference, and the two other groups were treated with 2000 and 5000 mg/kg of the extract. To study the behavioral changes, the treated rats were observed every 30 minutes for a period of two hours (23). The mortality of animals was recorded after 24 hours and the toxicity of the extract was determined.

Arterial thrombus formation time in vivo

In vivo arterial thrombus formation was studied as described previously (24) with a few modifications. Thirty rats of both sexes (170-250g) were divided into five groups of six rats. Animals were treated with aspirin (100 mg/kg), aqueous extract of fresh leaves of *Lagerstroemia speciosa* (250, 500, and 1000 mg/kg), and distilled water for five days. At the end of the treatment period, rats were anesthetized with ketamine (100 mg /kg). Dissection and isolation of the right carotid artery was operated to induce thrombosis with ferric chloride (35%). The formation of the thrombus was initiated by applying a saturated filter paper (5 × 10 mm) with ferric chloride to the carotid artery until the thrombus was formed. Ultrasound gel was deposited on the carotid artery and the blood flow was measured using an ultrasonic Doppler (BV-520 Vascular Doppler Detector, 9.0 MHz, Shenzhen

Bestman Instrument CO., LTD.) to follow the evolution of blood flow. The time taken to induce right carotid artery occlusion was measured up to 60 minutes.

Statistical analysis

Statistical analysis was determined by using one-way analysis of variance (ANOVA) and Turkey's multiple comparison test using GraphPad Prism 5 (GraphPad Software Inc., USA). The results were expressed as mean ± SEM (standard error of mean) of four independent measurements. The results were indicated significant at $P<0.05$.

Results

Phytochemical screening of aqueous extract of *Lagerstroemia speciosa*

The phytochemical study of the aqueous extract of fresh leaves of *Lagerstroemia speciosa* revealed the presence of sterols, polyterpins, flavonoids, polyphenols, and alkaloids. The extract did not contain saponins, quinones, and tannins.

Red blood cell protective activity of aqueous extract of *Lagerstroemia speciosa*

Hemolytic activity of aqueous extract of Lagerstroemia speciosa

Red blood cell of rabbit was used to assess hemolytic activity of EALS. EALS induced 10.5 ± 1.27% and 11.75 ± 1.2% of hemolysis in 10^{-3} and 10^{-1} mg/mL, respectively. Water (positive control) and saline solution (NaCl 0.9 %), used as a negative control, showed the hemolysis of 90.1 ± 10.6% and of 9.5 ± 0.8%, respectively. The percentage hemolysis of EALS was very close to that of saline solution (isotonic solution). The extract did not show any harmful effects on erythrocytes. Water is a hypotonic solution, its hemolysis percentage was significant in comparison with those of the saline solution and extract ($P<0.001$, n = 4) (Figure 1).

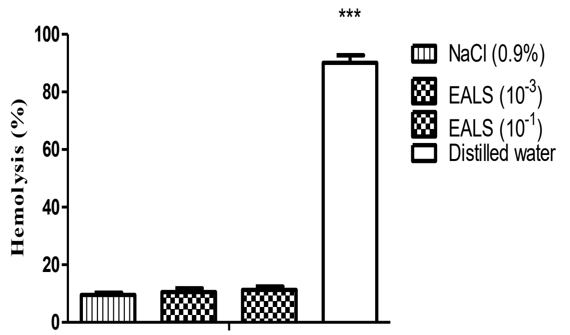

Figure 1. Hemolytic effects of the aqueous extract of *Lagerstroemia speciosa* (EALS), distilled water, and normal saline. EALS (10^{-3} and 10^{-1} mg/mg) hemolytic activity was very close of that of saline solution. Distilled water showed significant hemolysis effects in comparison with those of normal saline and extract (***$P<0.001$, n = 4).

Antihemolytic activity of aqueous extract of *Lagerstroemia speciosa*

AAPH was used to study antihemolytic activity of EALS. *Lagerstroemia speciosa* exhibited an antioxidant activity, thereby protecting erythrocytes from hemolysis. The percentage of hemolysis inhibition of EALS was increased in a concentration-dependent manner compared to that of ascorbic acid, which served as a positive control. Ascorbic acid showed significant inhibition of hemolysis ($P<0.001$, n=4). The antihemolytic effects of EALS and ascorbic acid are summarized in Figure 2.

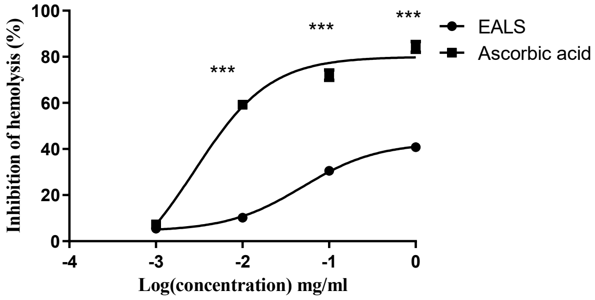

Figure 2. Antihemolytic effects of aqueous extract of *Lagerstroemia speciosa* (EALS) and ascorbic acid on 2,2-azobis hydrochloride-2-methylpropionamidine (AAPH)-induced hemolysis. EALS and ascorbic acid inhibited hemolysis. Difference between EALS and ascorbic acid values are significant (***$P<0.001$, n = 4).

Effects of aqueous extract of *Lagerstroemia speciosa* on blood coagulation and prothrombin time

The plasma poor platelet was used to study the effects of *Lagerstroemia speciosa* on PT and aPPT. The addition of increasing concentrations of EALS (10^{-3} -1 mg/mL) shortened PT, whereas high concentrations (2-4 m/mL) prolonged it. For concentration ranges between 10^{-3} to 1 mg/mL and 2 to 4 mg/mL, the PT extract/PT control ratio decreased from 1 ± 0.01 to 0.91 ± 0.08 and increased from 1 ± 0.01 to 1.3 ± 0.3, respectively. EALS effects was not significant on PT compared to the negative control (distilled water) ($P>0.05$, n = 4). Figure 3 shows EALS effects on PT.

Effects of aqueous extract of *Lagerstroemia speciosa* on activated partial thromboplastin time

EALS (10^{-3} -1 mg/mL) caused the decrease of aPPT in a concentration-dependent manner, while increasing concentrations ranging from 2 to 4 mg/mL increased it. For concentration ranges between 10^{-3} to 1 mg/mL and 2 to 4 mg/mL, the aPTT extract/aPTT control ratio decreased from 1 ± 0.01 to 0.70 ± 0.14 and increased from 1±0.01 to 1.8 ± 0.12, respectively. However, EALS effects were not significant on the aPPT compared to the negative control (distilled water) ($P>0.05$, n = 4). The outcomes are summarized in Figure 4.

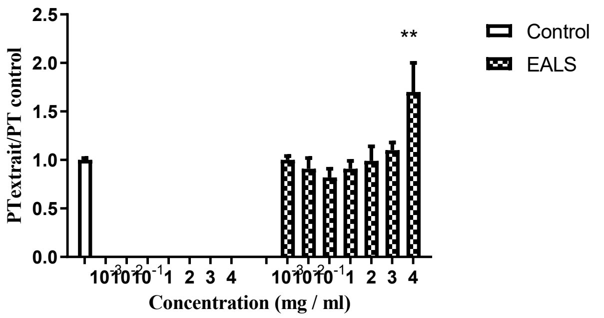

Figure 3. Effects of aqueous extract of *Lagerstroemia speciosa* (EALS) on prothrombin time *in vitro*. EALS showed coagulant effects at low concentrations (10-3-10-1 mg/mL) and anticoagulant activities at high concentrations (1-4 mg/mL) (*$P < 0.01$; n = 4).

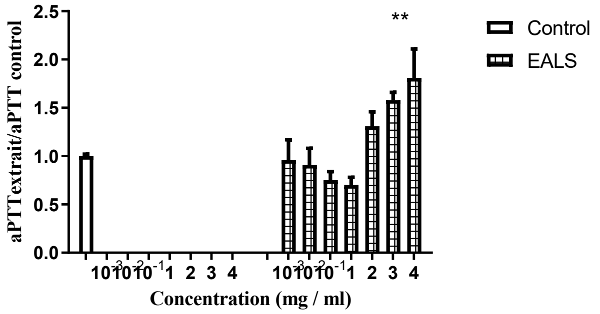

Figure 4. Effects of aqueous extract of *Lagerstroemia speciosa* (EALS) on activated partial prothrombin time (aPPT) in vitro. EALS showed coagulant effects at low concentrations (10-3-1mg/mL) and anticoagulant activities at high concentrations (2-4 mg/mL) (**$P < 0.01$; n = 4).

Thrombolytic effects of aqueous extract of *Lagerstroemia speciosa*

The thrombolytic activity of EALS was tested at the concentration of 4 mg/mL. At this concentration, EALS induced anticoagulant effects that promote thrombolytic activities. EALS and streptokinase showed significant thrombolytic activity compared to that of distilled water ($P < 0.001$, n = 4). The clot lysis levels of EALS, streptokinase, and distilled water were 49.5 ± 1.53%, 78.48 ± 2.2 %, and 5.7 ± 0.2%, respectively. Distilled water was used as negative control and streptokinase was chosen as reference.

Acute oral toxicity of aqueous extract of *Lagerstroemia speciosa*

EALS at the dose of 5000 mg/kg did not cause any mortality in rat. However, at the same dose, EALS induced a general modification of rat behavior compared to the control group. They became remarkably quiet, and remained in groups at the corner of the cage. Their spontaneous locomotive activity was reduced to 2 hours.

Effects of aqueous extract of *Lagerstroemia speciosa* on arterial thrombus formation in vivo

In this study, the rat carotid artery injury model was used to evaluate the effects of the aqueous extract of *Lagerstroemia speciosa* on arterial thrombus formation time. The thrombus was induced by ferric chloride (35%). After ferric chloride application, the injured vessels of the control group were occluded within 5.07 ± 1.35 minutes. EALS administered orally in increasing doses of 250, 500, and 1000 mg/kg dose-dependently prolonged the time to form a thrombus in the artery and occluded it, from 5.07 ± 1.35 minutes to 6.75 ± 1.05 minutes, 8.87 ± 1.02 minutes, and 16.22 ± 1.15 minutes, respectively. Acetylsalicylic acid (positive control) prolonged occlusion time from 5.07±.1.35 minutes to 18.07 ± 0.18 minutes. EALS (1000 mg/kg) and acetylsalicylic acid (100 mg/kg) increased the occlusion time significantly compared to that of distilled water, used as a negative control (**$P < 0.01$, n=4) (Figure 5).

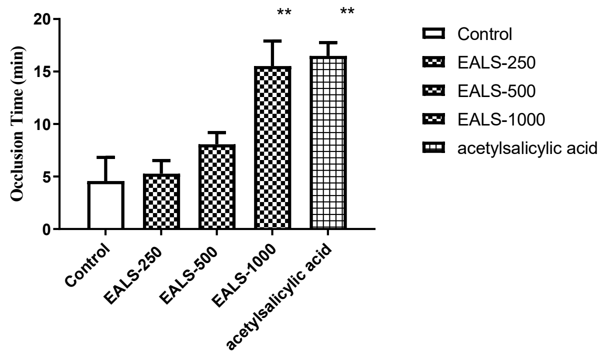

Figure 5. Effects of aqueous extract of *Lagerstroemia speciosa* (EALS) on occlusion time *in vivo*. EALS (1000 mg/kg) and acetylsalicylic acid (100 mg/mL) prolonged significantly occlusion time compared to that of distilled water (control) (**P <0.01).

Discussion

Antithrombotic activities of the aqueous extract of *L. speciosa* were evaluated using *in vivo* and *in vitro* tests. In *in vivo* experiments, EALS prolonged the occlusion time of thrombus formation when applied to a $FeCl_3$–induced thrombus formation model.

The likely mechanism of $FeCl_3$-induced thrombosis is oxidative vascular wall injury, causing endothelial damage and thrombosis. As another possible mechanism, micromolar levels of ferrous ions permeate the endothelium, and the ions in the bloodstream agglomerate with platelets, red blood cells, and plasma proteins, which subsequently induce thrombosis (25- 27).

To further confirm *L. speciosa* extract occlusion time prolonged effects, we evaluated its action on blood coagulation and on clot lysis *in vitro*. EALS showed coagulant effects at low concentrations and anticoagulant activities at high concentrations. Some plants also contain coagulant and anticoagulant constituents such as *Agastache rugosa* used to manage bleeding disorders (28). These kinds of natural products may play regulating actions to attenuate the anticoagulant effects induced at high concentrations in order to avoid bleeding complications associated with the use of the product.

Lagerstroemia speciosa significantly prolonged aPPT and PT at high concentrations, suggesting that the extract would act by inhibiting intrinsic and extrinsic pathways of blood coagulation. The anticoagulant effects could attribute to the presence of polyphenols in the extract, detected by phytochemical assay (29). The anticoagulant effects of *L. speciosa* revealed at high concentrations were very useful information before testing clot lysis activity. Indeed, anticoagulant effect promotes thrombolytic activity. Thus, the clot lysis test was performed at high concentration for 90 minutes. The decoction of the fresh leaves of *L. speciosa* exhibited strong thrombolytic activity *in vitro*. Similar results were obtained with dried leaf extract from *L. speciosa* in Bangladesh (11).

Hemolytic samples are reported to be unsuitable for coagulation testing (30). Thus, the ability of *L. speciosa* to protect red blood cells against hemolysis was elucidated in our work. EALS induced an antihemolytic property. The protective effects of EALS could be related to the presence of polyphenols in the extract (16). Acute oral administration of *L. speciosa* did not show harmful effects on treated rats. The decoction of fresh leaves of *L. speciosa* was not toxic.

Conclusion

The decoction of the fresh leaves of *L. speciosa* demonstrated antithrombotic and antihemolytic activities. Its antithrombotic properties are probably derived from anticoagulant and thrombolytic activities. A steady intake of *L. speciosa* leaves can prevent or treat thrombotic diseases. Further studies are necessary to isolate the anticoagulant components of *L. speciosa* and to evaluate its antiplatelet activities.

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Author's contributions

AV carried out statistics analysis. The first draft was prepared by KZF, and KBA supervised the work.

Conflicts of interests

The authors declared that there is no conflict of interests.

Ethical considerations

All authors examined ethical issues related to plagiarism.

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