In vitro Evaluation of Hemostatic Properties of Areal Parts Aqueous Extract of *Ageratum conyzoïdes* L. (Asteraceae)

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ABSTRACT

Ageratum conyzoïdes (Aerial part) is a medicinal plant commonly used in traditional medicine in Côte d'Ivoire in the treatment of various diseases including bleeding. The objevctives of this study were investigated the hemostatic effects of *A. conyzoïdes* aqueous aerial part extract at differents volume *in vitro*, using blood samples from apparently healthy human volunteers. Its properties have been identified by hematologic tests such as coagulation time, prothrombin and activated cephalin time. The results showed that the aqueous extract of *A. conyzoïdes* (100 mg/mL) significantly ($p < 0.001$) reduced clotting time, prothrobin time, and activated cephalin time compared to the control (administered with only NaCl 0.9 %) in the blood obtained from the volunteers. In contrast, the aqueous extract did not influence thrombocytes count ($p > 0.05$). The aqueous leaf extract of *A. conyzoïdes* in a volume of 0.5 mL in this study had coagulation properties, which indicates the positive hemostatic effect on *in vitro* method. In cconclusion, this study justifies its use to traditionally stop bleeding.

Key Words: *Ageratum conyzoïdes, Bleeding, Coagulant, Hemostatic effect.*

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INTRODUCTION

Hemostasis is the process of blood clot formation and shows a coordinated response to vessel injury [1]. It is accomplished by coordinating the efforts of 3 distinct but intimately related mechanisms : vascular, platelet, and coagulation phases of hemostasis [2]. Coagulation cascade occurs either through tissue factors (extrinsic pathway) or contact factors (intrinsic pathway) [3]. Increased coagulation is associated with several cardiovascular diseases, including coronary heart disease and hypertension [4, 5]. Cardiovascular diseases are the leading cause of death in the world due to blood coagulation disorders [6]. While decreased coagulation leads to prolonged bleeding time, which is results either from certain diseases such as haemophilia or from drugs like aspirin [7]. According to the WHO, approximately 80% of the population in Asia and Africa use traditional medicine as a form of health care to treat diseases that include amongst others blood disorders [8]. However, few plants have been studied for their hemostatic properties [9]. Therefore, the development of compounds to improve hemostasis is of medical importance. The ideal hemostatic agent needs to be inexpensive, easy to use, and capable of extended storage in a wide range of temperatures, ability to stop bleeding in 2 minutes and void of mixing or pre-application preparation [10]. Therefore, several authors demonstrated anticoagulant or coagulant effects of some plants on the examined blood samples [11]. Among the plants that are widely used for therapeutic purposes is the *Ageratum conyzoïdes* L. species. *Ageratum conyzoïdes* is a medicinal plant that is endemic to North and Central America with various indications in different diseases. The main uses of this

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plant in folk medicine are the treatment of colic, rheumatism, diarrhea, fever, wounds, and bleeding [12, 13]. Flavonoids, alkaloids, and tannins compose the main bioactive compounds of *A. conyzoïdes*. Once-daily administration of *A. conyzoides* methanolic leaf extract (250, 500, and 750 mg/kg) in albino rats for two weeks resulted in the dose-dependent decrease of bleeding time, prothrombin time (PT), and clotting time. Also, plasma fibrinogen concentration considerably is significantly increased in favor of hemostasis [13]. In addition, the ethanolic extract of *A. conyzoïdes* leaves was able to reverse the antiplatelet and anticoagulant effects of aspirin, clopidogrel, and enoxaparin combination in mice. Aspirin and clopidogrel are inhibitors of thromboxane A2, while enoxaparin is Xa factor and thrombin inhibitor of the intrinsic pathway. In the experimental groups that used 100 mg/kg and 250 mg/kg of ethanolic extract clotting time and the time of bleeding were significantly different from the negative control group (induction of drug combination only), and the 250 mg/kg dose was able to reduce these values close to the normal values (no drug administration) [14]. The results of both studies indicate the possible involvement of intrinsic and extrinsic pathways in the hemostatic activity of this medicinal plant [13, 14]. Referring to the recent toxicological safety assessment of this extract, effective hemostasis stimulating doses are safe. *A. conyzoïdes*, with 2000 mg/kg body weight/day no observed adverse effect dose, showed no toxicity or mortality in rats during the 90 days of repeated oral administration [15]. This study decided to evaluate the hemostatic effect of *A. conyzoïdes* aqueous leaf extract on human blood coagulation.

MATERIALS AND METHODS

Vegetal materiel

Aerial parts of *A. conyzoïdes* L. were harvested from Nangui Abrogoua University (Abidjan, Côte d'Ivoire) in september 2016. This plant was identified by us and authenticated in the laboratory of the botany of Nangui Abrogoua University. Fresh aerial parts were pulverized for use in the preparation of the aqueous extract.

Preparation of aerial parts of aqueous extract of A. conyzoïdes

Aerial parts of aqueous extract of *A. conyzoïdes* (AEAc) was obtained according to the method based on the traditional use of the plant by the people with slight modification. The fresh aerial parts were washed in tap water and pulverized employing an electric grinder (Culatti, France). One hundred grams (100 g) of fresh aerial parts were macered for 24 h in 1 L of distilled water under magnetic shaking. The obtained maceration was double filtered on hydrophilic cotton and Whatman filter paper (N°1). The extract was stored in capped bottles in the refrigerator at 4°C until required.

Human blood sample

Venous blood sample (5 mL) was taken from the elbow of each fasting from ten healthy adult volunteers of both sexes (aged 23 to 32), with no medication history for at least one week before blood sample collection. Subjects were recruited in July 2016 at general hospital of Abobo Sud (Abidjan, Côte d'Ivoire). This study was approved by the medical analysis laboratory of the general hospital of Abobo Sud. It allowed to include in this study the subjects healthy, non-smokers who were not on medication for at least a week. Children, pregnant women, women using contraceptives, and people suffering from pathology were excluded from the study.

Incorporation of an aqueous extract of A. conyzoïdes on blood samples

Initially, 0.3 mL of blood in the EDTA tubes was transferred to two tubes. Then, the first tube serving as control received 0.3 mL of physiological saline (NaCl 0.9%). The second tube serving as the test received 0.3 mL of aqueous extract of *A. conyzoïdes* (100 mg/mL). After 24 hours of incubation (37 °C), the thrombocyte of each tube was determined using an automatic hematological analyzer (Coulter Act Diff 2) [16].

The test was performed for each individual selected in this study (n = 10) to reduce any handling errors. The mean associated with a standard error on the mean (ESM) of 10 values obtained from these tests is used.

Tube coagulation time assay

In vitro Clotting time measurement was carried out using amodified method of Lee and White as reported these authors [17, 18]. The method described by some authors was adopted in this study with slight modifications.

For the tubes coagulation time assay, four tubes were used for the experiment. One tube served for control and another for the test. The test tube contained 0.5 ml, 1ml, and 2 ml, respectively, of aqueous extract of *A. conyzoïdes* (AEAc) at 100 mg/mL and the control test contained 0.5 mL of NaCl 0.9 %. Then, using a plastic syringe, 5 mL of venous blood was drawn from the donor, and the stopwatch was started as soon as the blood entered the syringe. 2 mL was added to each tube. The tubes were stoppered with carded cotton and immediately placed in a water bath (Fisher Scientific type, Polytest 20) at 37 °C. The test of every volume of AEAc was repeated (2 times) without shaking. After five minutes, the tubes were observed at 60-second intervals by tilting at a 45° angle to see if the blood was completely coagulated. Coagulation was considered complete when the tube could be reversed without significant movement of blood

in the tube. The time required for blood coagulation was recorded in each tube and the procedure was repeated for the other tubes.

Determine prothrombin time

This test consisted of measuring the coagulation time of a decalcified plasma after recalcification according to the method of [19] with a slight modification. 5 mL of venous blood is immediately collected in tubes containing EDTA. The blood was then centrifuged at 3000 rpm for 10 min at room temperature to obtain platelet-poor plasma and stored in a plastic tube. Then, 0.5 mJ of AEAc was divided into two tubes and two other tubes served as a control (Not received an extract). 100 µl of plasma were added to each tube. Thromboplastin was placed in a water bath at 37 °C. Finally, 200 µL of warmed thrmboplastin were forcibly added to the test plasma and the stopwatch was started. The timer started working as soon as the plasma entered the tube. After 5 min, the tubes were observed at 60-second intervals by tilting at a 45° angle to see if the blood was completely coagulated. Coagulation was considered complete when the tube could be reversed without significant blood movement in the tube. The time required for blood coagulation was recorded in each tube and the procedure was repeated for the other tubes.

Determination of activated cephalin time

5 mL of venous blood is immediately collected in tubes containing EDTA. The blood was then centrifuged at 3000 rpm for 10 min at room temperature to obtain platelet-poor plasma and it was stored in a plastic tube. Then, 0.5 mL of AEAc was divided into two tubes and the other two tubes were served as a control (unreceived extract). 100 µL of plasma and 100 µL of cephalin were added to all tubes. Calcium chloride was pre-warmed at 37°c for 5 min. Finally, 100 µL warmed of calcium chloride (0.025 M) were forcibly added to the test plasma and the stopwatch was started. It was finally added to the tubes. The timer started working as soon as the plasma entered the tube. After five minutes, the tubes were observed at 30 s intervals by tilting at a 45° angle to see if the blood was completely coagulated. Coagulation was considered complete when the tube could be reversed without significant blood movement in the tube. The time required for blood coagulation was recorded in each tube and the procedure was repeated for the other tubes [18]. All data were presented as mean ± SEM. Thrombocyte counts results were used by Student's t-test. Regarding coagulation time results, one-way analysis of variance (ANOVA) was used to analyze the data, followed by posthoc Tukey's multiple comparison analysis using GraphP software ad Prism 5.01 software for Windows **(**GraphPad Software Inc. San Diego, CA, California/USA, 2007). The results were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Determination of Thrombocyte counts

Table 1 showed the effect of *in vitro* aqueous extract of *A. conyzoïdes* (AEAc) at a dose of 100 mg/mL on thrombocyte counts. Addition of NaCl 0.9 % (Control) and AEAc (100 mg/mL) to the blood with 24 h of incubation at 37 °C, were 237.7 ± 1 5.62 10^3/µL and 231.6 ± 17.99 10^3/µL, respectively. The results clearly showed that there was no significant difference ($p > 0,05$) in the number of thrombocytes in the experimental group compared to the control group. The values always remain in the normal range (150 - 400 10^3/µL).

Table 1. *In vitro* effect of AEAc (100 mg/mL) on thrombocyte count

	Tubes	Control (NaCl 0.9 %)	Test (AEAc)	Reference
Thrombocyte counts (10^3/µL)	1	197	237	150 – 400
	2	256	200	
	3	199	160	
	4	346	342	
	5	289	302	
	6	237	213	
	7	219	187	
	8	201	238	
	9	189	179	
	10	244	258	
	M ± SEM	237.7 ± 1 5.62	23 1.6 ± 17.99 ^{NS}	

AEAc : Aerial parts aqueous extract of *A. conyzoïdes* ; NS : Not Significant ($p > 0.05$) vs control.

Determine the Clotting time

The mean clotting time in the control was 30.314 ± 1.8 29 min, while those of AEAc at 0.5, 1, and 2 mL were 22.403 ± 1.4 38, 26.156 ± 1.300, and 29.060 ± 0.934 min, respectively **(Table 2)**. There was a significant decrease ($p < 0.001$) of clotting time in AEAc at 0.5 mL compared to the control. While AEAc in 1 and 2 mL did not cause a significant difference ($p > 0.05$) in reducing clotting time compared to the control.

Table 2. Effect of *in vitro* aqueous extract of *A. conyzoïdes* on clotting time

AEAc (mL)	Clotting time in the tube (Min)
0 (Control)	30.314 ± 1.829
0.5	22.403 ± 1.4 38 ***
1	26.156 ± 1.300 ^{NS}
2	29.060 ± 0.934 ^{NS}

Values are expressed mean ± SEM ; n = 10 ; NS : NS : Not Significant ($p > 0.05$) vs control. *** $p < 0.001$ vs control. AEAc : Aerial parts aqueous extract of *A. conyzoïdes*.

Prothrombin time determination

The mean prothrombin time (PT) in the control group and AEAc at 0.5 ml were 14.00 ± 0.143 and 11.13 ± 0.167 s as shown in **Figure 1**. Also, a significant decrease ($p < 0.001$) of PT in AEAc at 0.5 ml was observed compared to the control.

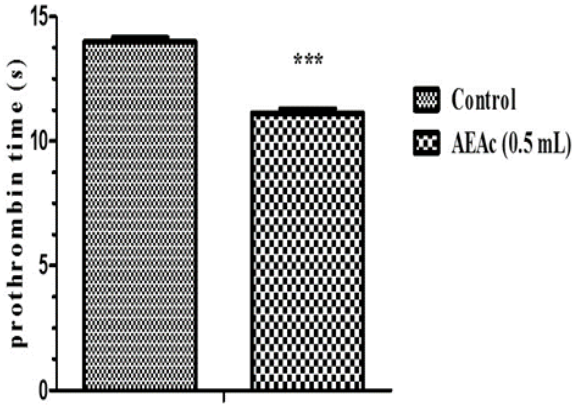

Figure 1. Effect of aqueous extract of *A. conyzoïdes* on prothrombin time.

Values are expressed mean ± SEM ; n = 10 ; *** $p < 0.001$ vs control. AEAc : Aerial parts aqueous extract of *A. conyzoïdes*

Activated cephalin time determination

In this study, the results showed that the mean activated cephalin time (aCT) in the control and AEAc at 0.5 mL were 27.29 ± 0.696 and 21.51 ± 0.637 s **(Figure 2)**. Also, compared to the control, a significant decrease ($p < 0.001$) of aPTT was observed in AEAc at 0.5 mL.

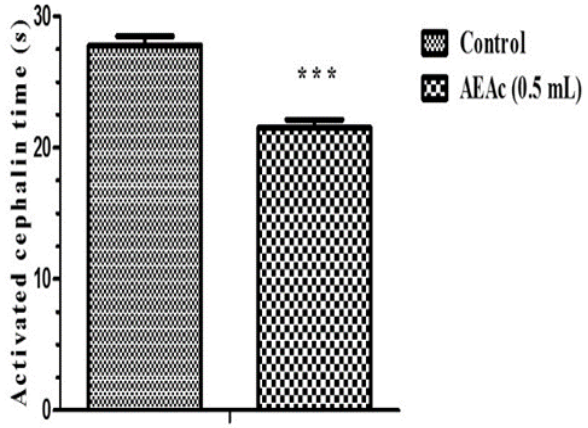

Figure 2. Effect of aqueous extract of A. conyzoïdes on activated cephalin time.

Values are expressed mean ± SEM ; n = 10 ; *** $p < 0.001$ vs control. AEAc : Aerial parts aqueous extract of *A. conyzoïdes*

The results showed that the thrombocyte counts was between 160 and 342 $10^3/\mu L$ after incorporation *in vitro* in a volume of 0.3 ml of aerial parts of aqueous extract of *A. conyzoïdes* (10 mg/mL) in blood samples. The values obtained were normal compared to the reference values. These results, on the one hand, indicate the absence of thrombocytopenia characterized by a significant decrease in thrombocytes ; and on the other hand, the absence of thrombocytosis due to a significant increase in thrombocytes compared to reference values (150-400 $10^3/\mu L$). Thrombocytes are the first elements to be activated primary hemostasis to stop bleeding due to vascular injury [20]. This lesion leads to the secretion of substances by thrombocytes and their aggregation with each other to stop bleeding [21]. Therefore, any quantitative or functional alteration in thrombocytes will be responsible for hemostasis disorders. This study can show some of the normal coagulation capacity of blood samples selected in this study. In addition, AEAc showed a non-significant decrease in thrombocyte counts compared to the control. Therefore, AEAc (100 mg/mL) did not affect thrombocytes in healthy subjects. This extract acts as a regulator of this parameter. This study showed that AEAc (100 mg/mL) at a volume of 0.5 mL significantly decreased clotting time compared to the control. However, AEAc in the volume of 1 mL and 2 mL does not affect this parameter. This reduction in clotting time depends on the volume of AEAc. Therefore, the aqueous extract in the volume of 0.5 mL has the property of accelerating coagulation. These results are related to the reports of some researchers about the haemostatic effect of methanolic leaf extract of *A. conyzoïdes* [13]. According to the authors, this extract arrested bleeding from fresh wounds by reducing both bleeding and clotting time. Clotting time test is a qualitative test to ensure the involvement of intrinsic factor. So, the reduction of clotting time until reach the normal time may be attributable to an increase in one or several intrinsic factors (I, II, V, VIII, IX, X, XI, XII) [13]. From the results, there is a significant reduction in clotting time, which indicates an increase in one or more clotting factors in the intrinsic pathway. The reduction in blood clotting time was confirmed by determining the prothrombin time and the activated partial thromboplastin time.

The results also showed a significant decrease in prothrombin time (PT). According to the author [22], PT test is used to investigate clotting blood from extrinsic and common pathways in which factors I (Fibrinogen), II (Prothrombin), V, VII and X contribute. The decrease in PT by AEAc is the result of an increase in the concentration of prothrombin or other extrinsic coagulation factors [23]. AEAc may have potentiated its effect on tissue factor activation. Similar results have

been reported by some researchers with methanolic leaf extract of *A. conyzoïdes* and aqueous leaf extract of *Chromolaena odorata* decreased during prothrombin time [13, 24]. Activated cephalin (aCT) was also significantly decreased from the results. This is a measure of the integrity of the intrinsic pathway. As the results point out the decrease in the activated cephalin time, it also suggests that there is an increase in one or more factors of the intrinsic pathway due to the presence of Vitamin K. The reduction in coagulation time as well as the prothrombin and activated cephalin times are attributed to the metabolites present in the aqueous extract of *A. conyzoïdes*. Some researchers demonstrated that astringent activity can be the presence of tannins respectively in the hydroalcoholic extract of the leaves of *Annona senegalensis* and an aqueous extract of *Marrubium vulgare* leaves [25, 26]. These two herbal remedies are used to treat bleeding. According to the same authors, astringent activity promotes vasoconstriction, which is an important parameter in hemostasis. The presence of several secondary metabolites and particularly of tannins, in an aqueous extract of *A. Conyzoïdes* leaves was indicated by another group of researchers [27, 28].

CONCLUSION

Aerial parts aqueous extract of *A. conyzoides* (AEAc) showed excellent effects in *in vitro* tests realized. AEAc at a concentration of 100 mg/mL effectively reduces clotting time, prothrombine time, and activated cephalin time. In fact, 0.5 ml of AEAc is the best volume to reduce these parameters. The plant material studied in this study is a credible alternative for an effective fight against bleeding and also promotes the coagulation of blood.

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Conflict of interest: None

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Ethics statement: Study was approved by the local ethics committee of Nangui Abrogoua University. The volunteers were given written informed consent forms which contain details about the research and contacts of investigators.

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