IJBPAS, February, 2017, 6(2): 193-209

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ANTIPLATELET, ANTITHROMBOTIC AND FIBRINOLYTIC ACTIVITIES OF *CHENOPODIUM ALBUM*

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Received 28th Aug. 2016; Revised 29th Sept. 2016; Accepted 25th Nov. 2016; Available online 1st Feb. 2017

ABSTRACT

Objective: The aim of the present work was to investigate the effects of *Chenopodium album* (CA) extract on antiplatelet, antithrombotic and fibrinolytic activities in rat and in human blood.

Methods: Rats were treated orally for 5 days with extracts and acetylsalicylic acid and at the end of the treatment period animals were challenged for bleeding and acute thromboembolism activity. In addition, we have assessed the prothrombin time and activated partial thromboplastin time (aPTT) after oral administration. In vitro studies, antiplatelet effects of different extract were evaluated on platelet aggregation, and fibrinolytic activity of the extract observed by rat or human artificial blood clot degradation. Platelet cytotoxicity of the extract was also determined by the LDH assay.

Results: Results demonstrated that CA has a significant protective effect on thrombosis. It also inhibits platelet aggregation without demonstrating cytotoxicity on platelets. CA slightly prolonged aPTT after oral administration. In addition, CA showed a fibrinolytic activity.

Conclusion: Thus, Our results have suggested that CA could be a very promising candidate for a cardiovascular protective agent.

Keywords: Activated partial thromboplastin time, prothrombin time, thrombosis, platelet, *Chenopodium album*, LDH, endothelium, thromboembolism

INTRODUCTION

Cardiovascular disease caused by a blood clot (thrombus) formation is one among the most severe diseases which are increasing at an alarming rate in the recent years [1]. Thrombolytic agents are used in the management of thrombosis [2]. Thrombolytic agents such as tissue plasminogen activator, Urokinase, Streptokinase etc are used all over the world for the treatment but their use is associated with hyper risk of haemorrhage, anaphylactic reaction and lacks specificity [3, 4].In the recent past, two decade's efforts have been carried out towards the exploration, discovery and designing and identification of natural products with antiplatelet, anticoagulant, antithrombotic and thrombolytic activity of the plants [5-7].

Traditional systems of medicine are popular in developing countries and up to 80% of the population relies on traditional medicines or folk remedies for their primary health care need [8].Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. Several plants such as *Ocimum sanctum*, *Curcuma longa*, *Azadirachta indica, Anacardium occidental [9], Molineria recurpata, Terminalia belerica, Tulbaghia violaceae, Curcuma longa, Melastoma malabathricum, Gloriosa superb, Jatropha curcas, Porana volubilis*, *Synclisia scabrida, Allium sativum Allium cepa* [10] have been proved to possess thrombolytic activity and many such plants are yet to be scientifically investigated. In the present study CA is used to evaluate the antiplatelet, thrombolytic activity etc. CA is one of herbaceous vegetable plant that has anthelminitic, laxative, diuretic, aphrodisiac action. It is also used in abdominal pains, eye diseases, throat troubles, piles, diseases of the blood, heart and spleen and biliousness [11]. However, as far as ascertained, no previous investigation was reported regarding antiplatelet and fibrinolytic activities of CA. Therefore, this study was carried out in rats to find out some useful or novel agent or lead compound for the treatment of cardiovascular diseases.

MATERIALS AND METHODS

Drugs and Reagents

Drugs used in the present study were acetylsalicylic acid (ASA), adenosine diphosphate (ADP), epinephrine and calf collagen type III used for experiments provided from Sigma-Aldrich (St. Louis, USA). Furthermore, streptokinase used in *in vitro* experiments was obtained from Merck (Germany). All other salts and chemicals were pure of analytical grade and obtained from Merck Chemical Company, Germany.

Plant and Extract Preparation

C. album is a cosmopolitan weed which is so widely distributed that its geographical origin is obscured. It is equally widely distributed in both the northern and southern hemispheres, occurring in Asia, North America, Europe [12] India, South Africa, Australia and South America [13].Fresh whole plant of *Chenopodium album* Linn (Family: Chenopodiaceae) was collected from the Soon valley of Punjab District Sargodha in July, 2014 and were then got identified and authenticated by a Taxonomist of the Botany Department, University of Sargodha, Sargodha. A voucher specimen (UOS 14/No.102) was deposited in the herbarium of Faculty of Pharmacy, University of Sargodha, Sargodha. Then the plant was shade dried and powdered finely with a Chinese herbal grinder and then powdered plant material was placed in the refrigerator at 4^{o}C.Aqueous- methanolic extract of whole plants of the *Chenopodium album* was prepared by cold maceration. For this purpose, 1 kg powdered plant material was soaked in 3L methanol and 1L of water for a week at room temperature. Following soaking for a week, it was filtered with muslin cloth. Then, filtrate mixture was concentrated with a rotary evaporator and filtrate was then air dried [14].

Animals and Human Participants

Both male and female Wistar rats weighing 250-350 g were used for the study. All the animals were housed in a controlled environment (23-25°C) and treated in accordance with the National Institute of Health (Pakistan) guidelines. The study protocol was approved by the local ethical committee of Faculty of Pharmacy, University of Sargodha and in accordance with NIH Publication No. 85-23, revised 1996.Besides, human blood was collected of six healthful individuals, with showed 30±7 years of age to conduct *in vitro* assays and informed consent was obtained from all the six subjects.

IN VITRO ASSAYS

Blood Manipulation

Blood samples were collected from rats or subjects into test tubes with sodium citrate 3.8% (1 part of citrate: 9 parts of blood). The samples of blood from rats were collected from the hepatic vein. The blood samples were centrifuged for 15 min at 180 ×g to obtain platelet-rich plasma (PRP) or 10 min at 2000 ×g to obtain platelet-poor plasma (PPP).

Platelet Aggregation Test

To evaluate the platelet aggregation, the test was performed by turbidimetric measurement with a Chrono-log optical aggregometer, with AGGRO/LINK Model 810-CA software for Windows version 5.1. After calibration of the aggregometer, the sample data concerning the assays and reagents were entered on a computer coupled with the equipment, and the sample test was then performed. Aggregation was recorded as the percent change in light transmission: the baseline value was set using PRP and maximal transmission using PPP. PRP was pre incubated at 37◦C for 4 min with the vehicle or the C. Album before addition of the platelet agonist, adenosine diphosphate (ADP). Maximal aggregation was obtained stimulating platelet with ADP 10 μM.

Determination of Cytotoxicity

To verify the possible effect of cytotoxicity of C. Album on platelets, we analyzed the leakage of lactate dehydrogenase (LDH) from platelets of human. After incubation with test drugs at 37◦C for 3 min, aliquots were collected and centrifuged at room temperature for 2 min at 12.000 ×g. A 50 μL aliquot of the resultant supernatant was used to measure the LDH leakage using enzymatic methods with (Labtest, Lagoa Santa-MG, Brazil). The extent of LDH leakage was expressed as % of total enzyme activity measured in platelets completely lysed with 10% sodium dodecyl sulphate (SDS final concentration was 0.05%).

Fibrinolytic Activity

The fibrinolytic activity of the *C. album* extract was observed by artificial blood clot degradation [15]. An artificial blood clot was made by spontaneous coagulation of 50 μL of fresh mice or human blood in a glass test tube. One hour later, the artificial blood clot was rinsed out repeatedly. The artificial blood clot was dipped in 1, 3, 10, 30, and 100 μg/ml of *C. album* extract or streptokinase at room temperature. Saline was used as a control. The degradation of the clot was estimated by color development after 1 h at room temperature. Then, red blood cells were lysed by adding 20 μL of triton 5% and then the absorbance of the final solution was read at 560 nm. The amount of color was estimated by linear regression analysis of a standard curve obtained from samples of mice blood properly diluted.

***In Vivo* Assays**

Animal Treatments

The rats were randomly assigned to distinct groups subjected to the following treatments: 10, 30, or 100 mg/Kg of *C. album* extract or 100 mg/Kg of ASA. Control animals received only vehicle. Pharmacological treatments were given orally once a day for 5 consecutive days. The last treatment was performed 3 h before the experiment. Animal body weights and general behaviour were recorded at the beginning and at the end of the subacute treatment to assess the tolerability of repeated administrations.

Bleeding

The tail transactions bleeding was determined applying the method modified[16].Briefly, tails of mice under light isofluorane anesthesia were transacted at 2 mm from the tip and immersed in 1 ml of 37 ◦C saline for 2 min. Red blood cells were lysed by adding 20 µL of triton 5% and absorbance of the solution was read at 560 nm. The amount of hemorrhage was estimated by linear regression analysis of a standard curve obtained from samples of mice's blood properly diluted.

Acute Pulmonary Thromboembolism

To verify the acute pulmonary thromboembolism, a modification of method[17]was used. Pulmonary acute thromboembolism was induced in mice by rapid intravenous injection in the tail vein of a mixture of 12 mg/Kg collagen and 1 mg/Kg epinephrine in order to induce about 85% of paralysis in the control group. The loss of the righting reflex for 30 s was considered as an indication of paralysis. The occurrence of paralyzed animals was recorded for 15 min after thrombotic mixture injection. Immediately after the last test, mice were euthanized by CO_2 chamber.

Effect on Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT)

For the measure of aPTT, citrate plasma (0.1 mL) was mixed with 0.1 mL of human placenta lipid extract (Pathrombin; Behringwerke), and the mixture was incubated for 2 min at 37 ◦C. Coagulation was initiated by the addition of 0.1 ml calcium chloride (25 mm), at which point the coagulometer was started and time to clot formation was recorded. For the measure of PT, citrated plasma (0.1 ml) was incubated for 1 min at 37 ◦C, at which point 0.2 ml of human thromboplastin (Thromborel; Behringwerke) was added. The coagulometer was started, and time to clot formation was recorded.

Statistical Analysis

Results were expressed as means ± SEM, except the gastric lesion scores that were expressed as median. Values of effective concentration that induce 50% of effect (EC_{50}) were reported as geometric means accompanied by their respective 95% confidence limits. Emax (maximal effect) and Imax (maximal inhibition) were calculated based on response of control groups. Data were analyzed by one-way analysis of variance (ANOVA), or t-test when appropriate. Post hoc tests (Student-Newman-Keuls test-SNK) were carried out when appropriate. Differences between groups were analyzed by χ2 test for pulmonary thromboembolism. Nonparametric Kruskal-Wallis followed by Dunn's test was used to analyze gastric lesion scores. The EC_{50} values were determined by nonlinear regression analysis using a sigmoidal concentration-response equation of individual experiments using GraphPad Software 5.0 (GraphPad, USA). $P < 0.05$ was considered indicative of significant differences between groups.

RESULTS:

Effect of *CHENOPODIUM ALBUM* extract on ADP-induced platelet aggregation:

We firstly evaluated the antiplatelet effects of *C. album* extract using freshly isolated human platelets against agonist-induced platelet aggregation. *C.album* extract showed a concentration-dependent inhibition of ADP-induced platelet aggregation. The Imax observed was 36 ± 5% of the concentration of 1000 μg/ml and the EC_{50} value of the *C. album* extract was 35 (15– 84) μg/ml compared with the control group (Figure 1 (a).

Effect of *C. album* on LDH release

To examine the cytotoxicity of *C. album* extract, we measured the LDH release from platelet for the determination of cell lysis. No concentration tested of *C. album* extract induced LDH release, while the positive control, SDS 10%, significantly increased the LDH release (Figure 1 (b) reflecting that *C. album* does not affect cell integrity.

Fibrinolytic activity of extract

In the evaluation of the fibrinolytic effect, blood clot degradation was observed in all the test tubes of *C. album* extract or streptokinase, with spreads of red blood cells trapped by multiple fibrins in rats and human blood. In rat blood, the EC_{50} value to *C. album* extract and streptokinase was 21 (5–87) μg/ml and 24 (9– 64) μg/ml, and the Emax observed was 56 ± 9% and 60 ± 14%, respectively, for the concentration of 100 μg/ml of both (Figure 2 (a)). In human blood, the EC_{50} value to *C. album* extract and streptokinase was 11 (3–33) μg/ml and 4 (1–10) μg/ml, and the Emax observed was 62 ± 7% and 70 ± 6%, respectively, for the concentration of 100 μg/ml of both (Figure 2 (b)).

Effect of extract on bleeding in rat:

The mean amount of blood lost from control animals 2 min after tail transaction was 5.8 ± 3.1 μL. Rats treated with antithrombotic doses C. Album, 30 and 100 mg/Kg/day, showed a significant difference from control, losing 24.9 ± 5.9 and 42.9 ± 15.7 μL of blood, respectively. ASA-treated animals lost 51.9 ± 12.9 μL of blood, showing a significant difference when compared to the control group (Figure 3).

Effect of extract on pulmonary thrombosis in rat

C.album had highly significant antithrombotic activity when administered at 100 mg/Kg day since it prevented paralysis induced by events in 80% compared with the control. ASA displayed lower efficacy since it showed no significant difference compared to the control to reduce paralysis (Table 1).

Effect of extract on a PTT and PT

As shown in Table 2, the aPTT and the PT in the control group were 29.99 ± 0.23 sec and 10.99 ± 0.03 sec, respectively. In the *C. album*-treated groups, the aPPT increased significantly to 31.89 ± 0.24 sec and 33.91 ± 0.34 sec, at the doses of 30 and 100 mg/Kg, respectively. There were no significant increases of PT when compared to the control group (Table 2).

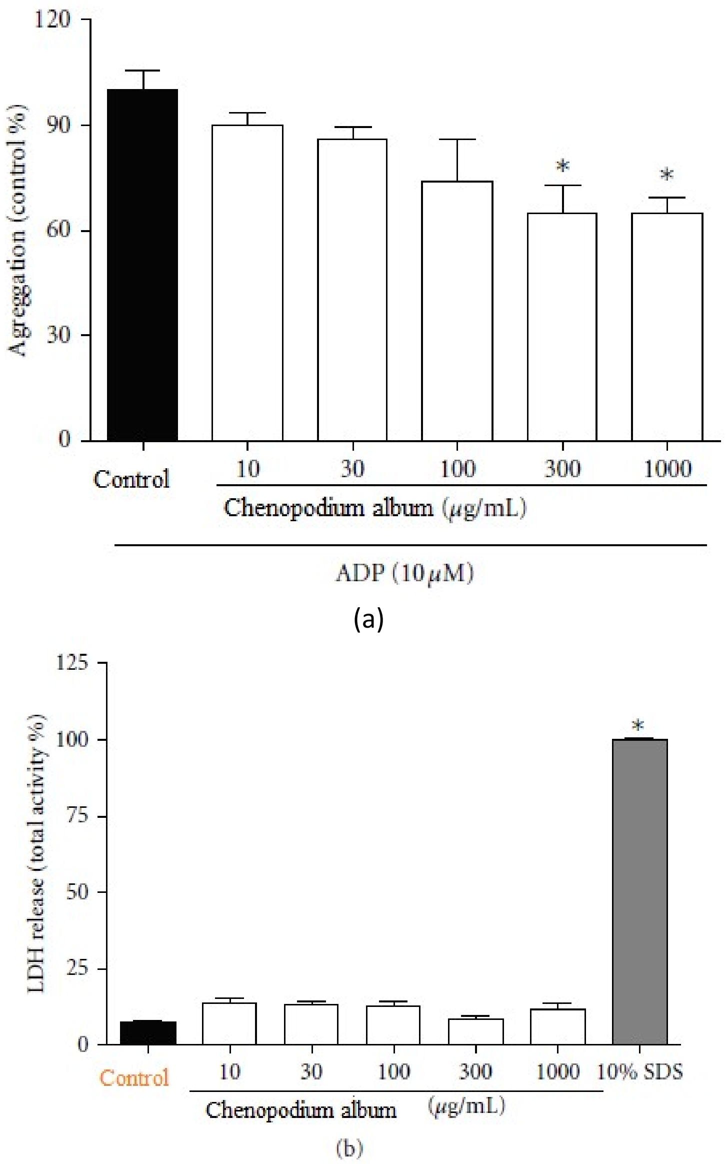

Figure 1: Effects of *Chenopodium album* extract on ADP induced platelet aggregation (a) and on LDH release in platelets (b). Data are shown as mean ± S.E.M. (n = 4–6). *P < 0.05 versus vehicle control (one-way ANOVA, followed by Student Newman- Keuls' test)

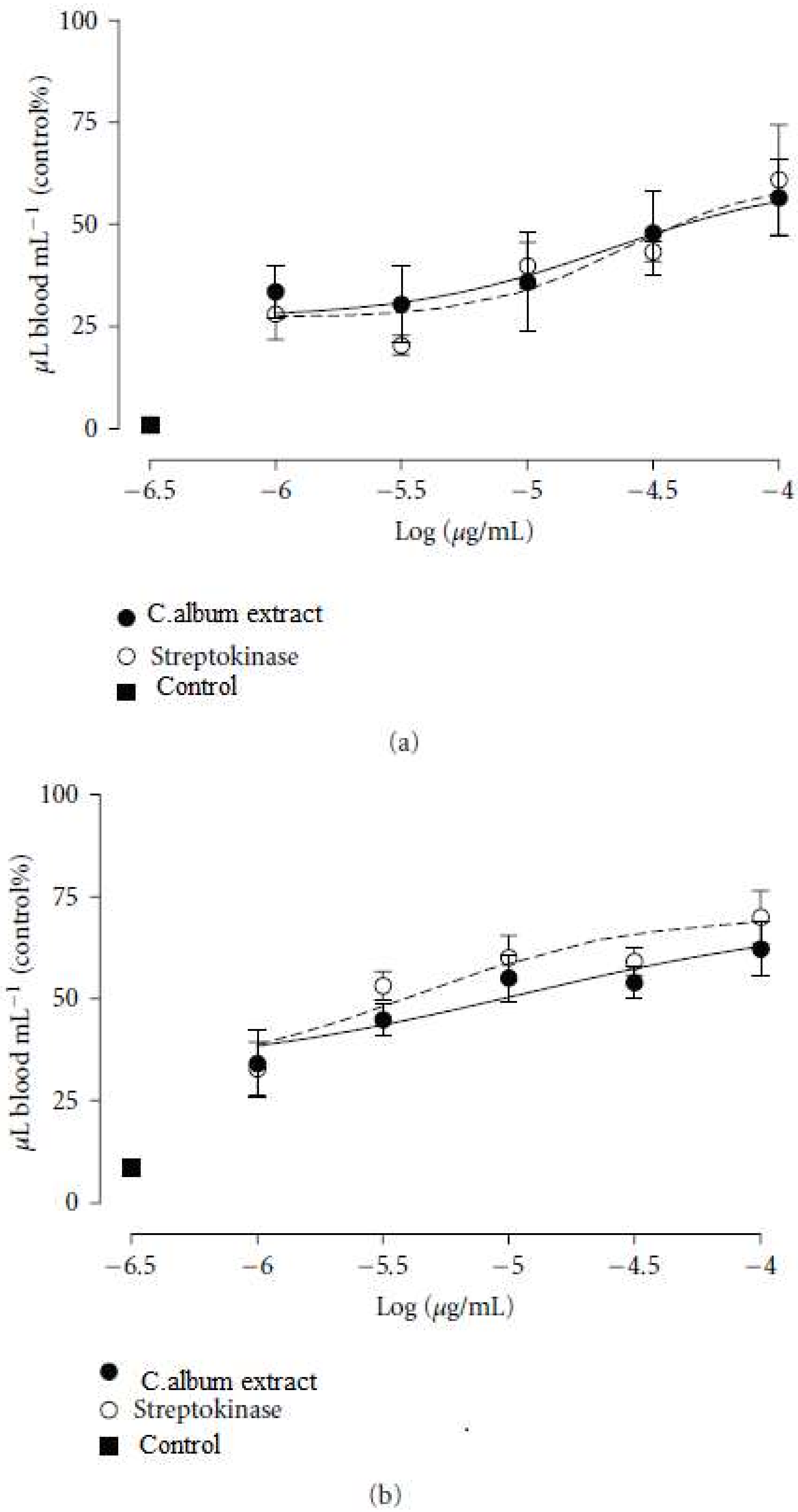

Figure 2: Effects of *Chenopodium album* extract or streptokinase on fibrinolytic activity. Concentration-response curve to rat (a) or human (b) blood. Each point represents the mean of 3 experiments performed in duplicate and the vertical lines represent SEM (nonlinear regression analysis).

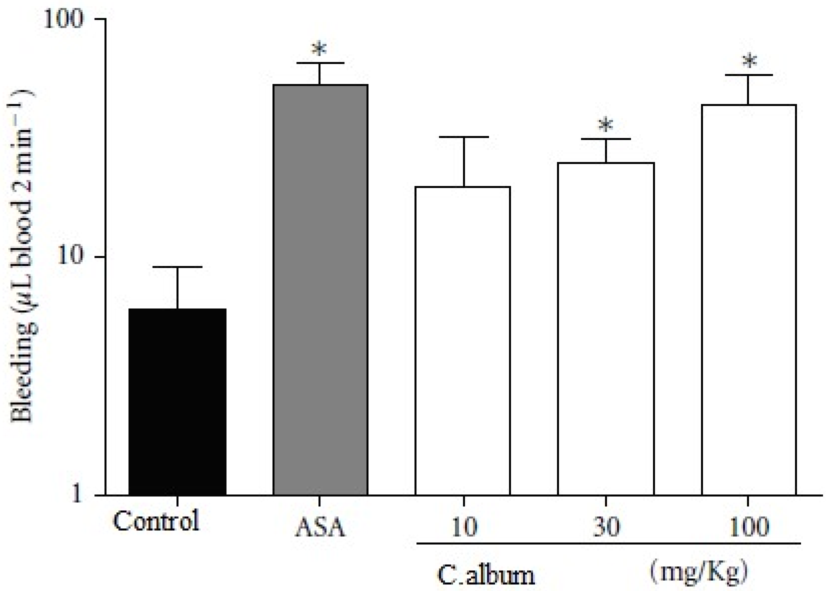

Figure 3: Effects of *Chenopodium album* extract or ASA on bleeding in rat. Data are shown as mean ± SEM (*n* = 4). **P* < 0.05 versus vehicle control (one-way ANOVA, followed by Student Newman-Keuls' test).

Table 1: Effects of *Chenopodium album* extract on pulmonary thrombosis in rat

Treatment	Dose (mg/kg)	No. of paralysed/ no. of treated rats	Protection (%)
Control	____	8/10	10
Chenopodium album	10	6/8	33.3
	30	5/10*	66.6
	100	2/10*	80
Aspirin	100	5/9*	55.5

****P* < 0.05 versus control. Data are shown as mean ± SEM (*n* = 9-10) ($\chi 2$ test).**

Table 2: Effect of *Chenopodium album* extract on *ex vivo* aPTT and PT in rat

Treatment	Dose (mg/kg)	aPTT (sec)	PT (sec)
Control	____	29.99±0.23	10.99±0.03
Chenopodium album	10	29.95±0.01	10.91±0.12
	30	31.89±0.24*	11.11±0.14
	100	33.91±0.34*	11.99±0.21

****P* < 0.05 versus vehicle control (one-way ANOVA, followed by Student Newman-Keuls' test). Data are shown as mean ± SEM (*n* = 6).**

DISCUSSION

The aim of the present work was to investigate the effects of *Chenopodium album* (CA) extract on antiplatelet, antithrombotic and fibrinolytic activities in rat and in human blood. Results demonstrated that CA has a significant protective effect on thrombosis. It also inhibits platelet aggregation without demonstrating cytotoxicity on platelets. CA slightly prolonged aPTT after oral administration. In addition, CA showed a fibrinolytic activity, previously same results of hwanggeumchal sorghum were reported by Kim (2013) [18] in vitro. Platelets are blood cells that participate in the human primary hemostatic mechanism. Platelet-platelet interaction has the final purpose to produce a platelet thrombus that constitutes the primary hemostatic plug

[19]. In addition, platelet adhesion and aggregation on blood vessel walls contribute to the occurrence of thrombosis and emboli formation, and have relation with other cardiovascular diseases [20-22]. Thus, it is important to evaluate platelet function in the thrombosis. Several studies have been carried out to develop antithrombotic agents with improved efficacy for preventing or treating arterial or venous thrombosis [23, 24]. Here, we evaluated the effect of *C. album* on platelet aggregation, as well as its antithrombotic and fibrinolytic activities that are still unknown. The results of the present study demonstrated that *C. album* extract has a significant protective effect on thrombosis once it is capable of inhibiting platelet aggregation without demonstrating cytotoxicity. Furthermore, *C.album* extracts, slightly prolonged apt, showed a fibrinolytic activity after oral administration. The interactions between platelets and various adhesive proteins, such as collagen, and soluble agonists, such as ADP, provide potential targets for developing antiplatelet agents [25]. The effect of *C. album* extract on hemostasis in in vivo studeis was studied using a model of pulmonary thromboembolism induced by the intravenous injection of collagen plus epinephrine. This model is characterized by the massive activation of circulating platelets and the widespread formation of platelet thrombi in the microcirculation of the lungs, leading to disseminated pulmonary microembolism and paralysis of the animal [26]. The oral administration of *C. album* extract, once a day for 5 days, markedly reduced the percentage of paralysis in a fair dose-dependent manner. *C. album* extract has been proved to be more effective than ASA in preventing paralysis. During thrombus formation, ADP plays a key role inducting the interaction of membrane receptor glycoprotein IIb– IIIa with fibrinogen, being the most important aggregating agent [27, 28]. Thus, ADP was chosen as agonist to induce platelet aggregation in the study. Results showed that during platelet aggregation, the *C. album* extract significantly inhibited ADP-induced platelet aggregation that was in line with previous studies [29, 30, 31]. Inhibitors (anticoagulants) and activators (procoagulant) of blood coagulation may affect any of the factors. The PT test and the aPTT test are used for distinguishing between the effects of test agents on the extrinsic and intrinsic pathways. In the clinical tests of blood coagulation, applet is used to evaluate the intrinsic clotting index. A prolonged aPTT usually represents a deficiency in factors VIII, IX, XI, XII, and V or Willebrand's factor. PT is used to

evaluate the extrinsic clotting pathway. A prolonged weak PT indicates a deficiency in coagulation factors V, VII, and X [32]. The results reported here show that the *C. album* did not have an anticlotting effect when examined by the PT test, a slight anticoagulant effect is portrayed by the apt test. This indicates that the *C. album* might not inhibit a factor or factors in the intrinsic pathway of blood coagulation. At this stage, there is no indication of the presence of anticoagulants or procoagulants components in the *C. album*, since it did not affect the PT. Although *C. album* has shown a slightly anticoagulant effect on aPTT test, this effect did not show clinical signs because the values remained in normal limits that were in line with [33, 34]. To further assess the possible effect of C. Album on platelet activity, the bleeding in rat was measured. The evaluation of bleeding is a crude test of homeostasis, which is a useful tool to estimate how well platelets interact with blood vessel walls to form blood clots [35]. Here, we demonstrated that *C. album* extract treatment and ASA affect blood loss. The transactional bleeding time in rat model is sensitive not only to platelet-active compounds, but also to drugs that are capable of influencing the fibrinolytic system [36]. Besides, the treatment with *C. album* extract was well tolerated since the treated rats grew similarly to control rats (data not shown). The fundamental task of thrombolytic or fibrinoytic therapy is the degradation of fibrin by placement, which can be activated by plasminogen [37]. However, there are findings of bacterial contaminants of plants which have plasminogen receptors that bind plasminogen. Few plant extracts and their products having fibrinolytic activity are identified, which includes *Lumbricus rubellus* [38], *Pleurotus ostreatus* and *Spirodela polyrhiza*, Ginger (*Zingiber officinale*), Garlic (*Allium sativum*) [39, 40]. Urokinase which was derived from human urine has been broadly employed for thromboembolism therapy, but it has low specificity to fibrin and high cost. The fibrinolytic enzyme prevents the formation of fibrin clot in the circulatory system. Some medicines like urokinase and streptokinase are widely used to inhibit hemostatic disorders, particularly thromboembolism [41]. We also investigated the fibrinolytic activity of the *C. album* extract in human and in rats and then compared to streptokinase. To further investigate the possible mechanism of action of *C. album*, streptokinase was used as standard. Probably, *C. album* shows the same mechanism similar to the streptokinase. We suggest that the primary role of *C. album* is the conversion of plasminogen to plasmin. Activated plasmin enzymatically cleaves fibrin, which, with platelets and other hemostatic elements, underlies the pathological processes of the acute occlusive disorders [42]. Besides, when placement breaks down fibrin, a number of soluble parts are produced as the D-dimmer, that is, a small protein fragment present in the blood after a blood clot is degraded by fibrinolysis [43]. Preliminary results of our group demonstrated that both *C. album* and streptokinase produced D-dinner during fibrinolytic activity as earlier demonstrated by [44] Klafke et al., 2012. These optimistic results pointed the value of further studies in this field. Then, with the results documented in the paper we could only assume that *C.album* might have a similar mechanism of streptokinase, and the result suggested that the *C.album* extract exerted its antithrombotic activity besides inhibiting platelet aggregation, through fibrinolytic activity. Antithrombotic agents acting in only one pathway of thrombosis formation have limited efficacy in treating arterial thrombotic diseases [45]. Hence, the *C. album* extract with a combination of antiplatelet aggregation, antithrombotic, and fibrinolytic activities may be effective in preventing thrombus formation through several pathways. Saponins are widely distributed in plants and have many biological activities, such as antiplatelet activity [46, 47]. The phytochemical components responsible for *C.album* antiplatelet effect are still unknown, but preliminary studies demonstrated the presence of alkaloids and flavonoids (quercetin, Kaempferol, rutin) in this plant [47,48]. Moreover, recent preliminary phytochemical analysis of *C.album* indicated the intense presence of saponins (calenduloside,chikusetsusaponin), tannins, and turbines and small presence of flavonoids [49] that might be responsible for such activities. Thus, we suggest that *C. album* bioactive compounds may be inhibiting platelet aggregation.

CONCLUSION

In conclusion, CA strongly inhibited platelet aggregation in ADP activated platelets and In addition, CA effectively decreased the paralysis of rat from thromboembolism as compared to aspirin. Our results suggest that CA could be a very promising candidate for a cardiovascular protective agent.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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