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In vitro anticoagulant effect analysis of leaves different extract types of *Calotropis procera* (Asclepiadaceae) in West Bank/ Palestine

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ABSTRACT

As several plants have anticoagulant activity, *Calotropis procera* has been investigated *"in vitro"* in this study. Hot and cold water, ethanol, and methanol extracts of these plant leaves were prepared to final concentrations of 100, 50, and 25 mg/ml. *In vitro,* PT and aPTT assays were conducted on normal platelet poor plasma blood samples of 20 healthy volunteers by a digital coagulation analyzer. Results showed that all evaluated extracts prolonged both PT and aPTT at 100 and 50 mg/ml concentrations. The highest effect on PT was observed for ethanol and methanol extracts by recording 128.52±24.45 sec and 105.99±17.76 sec at 100mg/ml concentrations, respectively. The high effect on aPTT was observed for all evaluated extracts by recording 420 ±0.00 sec at 100mg/ml concentration. So they could have an inhibitory effect on the clotting factors in the intrinsic and extrinsic pathways and those in the common pathway. While at 25 mg/ml concentrations, they prolonged PT only, 16.63±1.78 sec, 15.34±0.97 sec, 16.19±1.32 sec, and 16.02±1.37 sec for hot water extract, cold water extract, ethanol extract, and methanol extract, respectively. Suggesting their inhibitory effect was on the extrinsic pathway tissue clotting factors. On the contrary, they demonstrated a decreasing effect on aPTT at a concentration of 25 mg/ml, recording zero aPTT, affecting the intrinsic pathway. Moreover, compared with the positive heparin control, only the alcoholic extracts at 100 mg/ml concentration showed a similar anticoagulant effect on PT (P>0.05). While all evaluated plant extracts at a concentration of 100 mg/ml revealed similar bioactivity on aPTT (P>0.05). Still, all evaluated extracts' anticoagulant and procoagulant activity need *in vivo* clarification.

Keywords: *Calotropis Procera*; Plant Extract; Anticoagulant; PT; Aptt.

INTRODUCTION

Blood is a vital tissue that consists of a mixture of cells (red blood cells, white blood cells, and platelets) and plasma. The smooth flow of blood is essential for its physiological functions. However, still, the process of blood clotting is equally important. Blood clotting is a normal and necessary process because it helps prevent loss of life, even from minor injuries, to stop bleeding from a damaged or injured vessel [1], and maintains hemostasis [2] by forming clumps or clots. Therefore, under physiological conditions, blood clotting is an important part of hemostasis [1], in which vessel wall, platelet function, coagulation factor cascade, and clot inhibition/fibrinolysis are its major components. They work together to prevent prolonged bleeding or thrombosis [3]. However, insufficiency in this process can lead to excessive or spontaneous bleeding, such as hemophilia; in contrast, excessive blood clots can induce thrombosis and fatal embolism [4].

Plant products have been widely utilized as effective therapeutic tools for treating diseases and injuries [5]. Since plants are rich sources of variable concentrations of biologically active compounds in one or more different parts of the plant [6], traditional medicine has been used as a form of healthcare for treating several diseases, such as blood disorders [2]. This may be due to their incredible pharmacological activities, economic viability, and fewer side effects [7]. Moreover, increased demand for natural products and their derivatives in developing and developed countries has increased interest in medicinal plants as an alternative medicine [8]. Therefore, according to the word health organization (WHO), approximately 80% of the world's population uses traditional medicine to treat several diseases [9]. It is reassuring

that there is compelling scientific evidence providing the risk of thromboembolic diseases, which could be reduced or eliminated by consuming dietary anticoagulants or phytochemicals with anticoagulant properties [10-12]. As several plants have anticoagulant / antithrombotic activity, they are used for treating thromboembolic diseases in different systems of traditional medicine [12]. Some plants have latex proteases, which act as procoagulants [13,14], interfering with blood coagulation and fibrin hydrolysis [14,15]. Also, plant extracts have bioactive chemicals such as alkaloids, coumarins, flavonoids, anthraquinones, and naphthalenes. These compounds affect platelet aggregation. For these reasons, plant extracts can be substituted for the currently used anti-platelet agents [9].

In Palestine, studies focused on active biological properties of different plant extracts such as antitumor, antibacterial, antifungal, and antioxidant of wild plants [16]. However, limited studies on the anticoagulant properties of wild plants were reported. For example, *Viscum album* extracts from Palestine's olive and almond host plants significantly prolonged PT and aPTT [17]. Also study on the bioactivity of eight wild plants on the coagulation cascade showed that some of the studied plant species extracts possess potent anticoagulant properties [18].

Calotropis procera (Aiton) Aiton f. (Asclepiadaceae) is one of the wild plants present in Jericho/ Palestine. *Calotropis procera* is known as "aak" in India and as "Sodom apple" or "usher" (swallow-wort in English and Akundiu in Hindi). It is widely distributed in West Africa and other parts of the tropics, and it grows abundantly in arid and semi-arid regions without irrigation, chemical fertilizers, pesticides, or other agronomic practices. *Calotropis procera* is well known for its high medicinal properties, so it is considered a golden gift for humans [5]. It is used alone or with other herbs to treat common diseases such as fevers, eczema, cold, indigestion, rheumatism, eczema, and diarrhea [19]. The latex of *C. procera* possesses various pharmacological properties that may be used to treat inflammatory disorders such as arthritis, cancer, and sepsis [20] and cutaneous diseases such as ringworm, leprosy, and syphilitic sores [19]. Also, it exhibits procoagulant activity [21].

Furthermore, the secretions from the root bark are traditionally used to treat intestinal worms, skin diseases, and enlargements of abdominal viscera. In addition, leaf extracts, chopped leaves, and latex of *C. procera* have shown great promise as a nematicide *in vitro* and *in vivo* [19]. However, all published studies were related to the anticoagulant effect of the latex of *C. procera*. For example, the derived latex protein (LP) of *C. procera* produced a procoagulant effect when intraperitoneal injection of LP was performed on normal mice. In addition, a subfraction of LP (LPPI, LPPII, and LPPIII) produced both fibrinogenolytic and fibrinolytic effects that were mediated through the hydrolysis of the Aα, Bβ, and γ chains of fibrinogen and α-polymer and γ-dimer of a fibrin clot, respectively [21]. Another study on the latex cysteine peptidases in C. procera showed that all peptidases exhibited fibrinolytic activity and reduced clotting time by 50% [20].

Nevertheless, the data available on the validity of wild plant use in Palestine for thromboembolic disease or wound healing is rare. At this point, more studies were suggested on the hemostatic bioactivity of other wild plant species, one of which is *Calotropis procera.* The previous information was behind the search target of analyzing *in vitro* the anticoagulation effect of hot and cold water, ethanol, and methanol extracts of *C. procera* leave in West Bank/Palestine. This was conducted by analyzing healthy volunteers' blood samples' activated partial thromboplastin time (aPTT) and prothrombin time (PT).

METHODS

Plant materials

All plant samples of *C. procera* were collected in April 2016 from Jericho in Palestine. The collected plants were identified by Dr. Ghadeer Omar, An-Najah National University; Palestine, Department of Biology & Biotechnology. Representative plant specimens of the examined plant species were collected, pressed till drying, treated chemically, mounted on herbarium sheets, and provided with voucher numbers (1852), and then they were deposited at the An-Najah National

University herbarium. The leaves of the plant were washed to eliminate soil and dust particles, then air dried. Light exposure was avoided to minimize or prevent possible loss of active molecules. The air-dried leaves were powdered using a grinder and stored at room temperature until they were used.

Equipment

Grinder (MÜLLER), Orbital shaker (YIH DER Technology Co., Ltd), Centrifuge (Universal R320R Health), Rotary evaporator (Bibby Scientific), Shaking incubator (Lab Tech LS1-3016A), Freeze drier (Millrock Technology, Inc.) and Digital coagulation analyzer (Coa DATA 4004, LAber Biomedical Technologies, Germany).

Chemicals and reagents

The following chemicals and reagents were used: methanol, and ethanol (Merk), DMSO (dimethyl sulfoxide) (Sigma-Aldrich, Germany), citrated tubes (Greiner Bio-One), Heparin (Sigma-Aldrich, Germany), PT reagent (Hemostat thromboplastin-SI. Human, Germany), aPTT reagent (Human, Germany) and calcium chloride solution ((Human, Germany).

Extraction procedure

Ethanol and methanol extractions

Ten grams of plant leaves powder were soaked in 100 ml of 70 % ethanol and 10 g in 100 ml of 70% methanol and incubated in an orbital shaker (120 rpm) at room temperature for one week. Then the mixtures were centrifuged for 5 min at 5000 rpm. A rotary evaporator evaporated the supernatants. The obtained powder of the examined plant was dissolved in 1% dimethyl sulfoxide (DMSO) to final concentrations of 100, 50, and 25 mg/ml [18].

Hot and cold water extractions

For hot extraction, 10 g of plant leaves powder were soaked in 100 ml of boiled sterilized distilled H_2O at 100ºC and incubated in a shaking incubator at 37ºC for one week. Then the mixtures were centrifuged for 5 min at 5000 rpm. The supernatants were dried with a freeze drier. The obtained powder of the examined plant was dissolved in cooled sterilized distilled H_2O to final concentrations100, 50, and 25 mg/ml. For cold water extraction, the procedure used is the same as hot water extraction, but plant powder was soaked in 100 ml of sterilized distilled H_2O at 20ºC and incubated in an orbital shaker at room temperature (25ºC) [18].

Blood Sample Preparation

Twenty healthy volunteers (18-52 years old, 57-93 kg weight) of both genders (10 males and 10 females) were asked to give blood samples. The volunteers should not be under any medication and not be smokers. The blood samples were collected in citrated tubes (9 parts of blood were mixed with 1 part of 3.2% sodium citrate solution). The samples were processed as the following; each blood sample was centrifuged at 3000 rpm for 15 min to obtain the Platelets Poor Plasma (PPP) [22]. All samples were subjected to PT and aPTT assays within 2 hours after blood collection. Clotting time for both tests was recorded by a digital coagulation analyzer (Coa DATA 4004, LAber Biomedical Technologies, Germany). All coagulation assays were performed in duplicate. Heparin (0.016 mg/ml) was used as a positive control for PT and aPTT assays. At the same time, 1% DMSO and sterilized distilled H_2O were used as the negative controls for the alcoholic and aqueous extracts, respectively.

Prothrombin Time (PT) assay

For *in vitro* PT assays, 50 μl normal citrated blood sample (PPP) was incubated with 50 μl from each plant extract at different concentrations (100, 50, 25 mg/ml) for 5 min at 37°C. The PT clotting time was immediately recorded after adding 100 μl PT reagent (Hemostat thromboplastin-SI. Human, Germany) [18].

Activated Partial Thromboplastin Time (aPTT) assay

For *in vitro* aPTT assays, 50 µl normal citrated blood sample (PPP) was incubated with 50 µl from each plant extract at different concentrations (100, 50, 25mg/ml) for 2 min at 37°C.Then 50 μl aPTT reagent (Human, Germany) was added and incubated for 3 min at 37°C. The aPTT clotting time was immediately recorded after adding 100 µl calcium chloride solution (Human, Germany) [18].

Statistical analysis

Statistical analysis of PT and aPTT mean value results were conducted using a statistical package SPSS (Version 20) t-test for independent samples to determine if there was a significant difference among different examined plant extracts relative to the control. The *P*-value ≤ 0.05 was considered to be significant. This statistical analysis was conducted by Dr. Ali Barakat, An-Najah National University, Faculty of Science, Department of Statistics.

RESULTS

The anticoagulation results of the examined hot water, cold water, ethanol, and methanol extracts of *C. procera* showed no individual variation among the examined blood samples for both PT and aPTT (*P*>0.05). Therefore, they could be considered a representative sample for this study. In addition, the negative controls (1% DMSO and sterilized distilled H_2O) in the PT and aPTT analysis in this study showed no significant effect relative to the normal control (*P*>0.05).

All evaluated extract types of *C. procera* at all studied concentrations (100, 50, 25 mg/ml) increased PT significantly (*P*≤0.05) relative to the normal control values. Moreover, compared with the positive heparin control, only the ethanol and methanol extracts at 100 mg/ml showed a similar anticoagulant effect (P>0.05), while other samples had less anticoagulant effect than heparin. Recorded data of the evaluated plant extract types at the different investigated concentrations on PT are represented in (Table 1 and Figure 1).

Table (1): The PT values of the studied *Calotropis procera* extracts at 100, 50, and 25 mg/ml concentrations.

Plant extracts	Concentration (mg/ml)	PT±SD[a] (sec)	* *P*-value	** *P*-value
Hot water	100	77.17±12.98	0.000	0.069
	50	28.57±7.50	0.000	0.001
	25	16.63±1.78	0.000	0.000
Cold water	100	50.66±6.66	0.000	0.007
	50	22.16±1.98	0.000	0.000
	25	15.34±0.97	0.000	0.000
Ethanol	100	128.52±24.45	0.000	0.895
	50	27.20±2.56	0.000	0.001
	25	16.19±1.32	0.000	0.000
Methanol	100	105.99±17.76	0.000	0.436
	50	26.41±2.87	0.000	0.001
	25	16.02±1.37	0.000	0.000
Normal control (blood sample without plant extract)		12.76±0.78		
Negative control (sterilized distilled H_2O)		12.58±0.83		
Negative control (1%DMSO)		12.35±0.95		
Positive control (Heparin = 0.016 mg/ml)		127.21±106.95		

**P*-value ≤0.05 is significant [between the different extract types investigated concentrations relative to the normal control].

***P*-value ≤0.05 is significant [between the different extract types investigated concentrations relative to the positive control (heparin= 0.016 mg/ml)].

SD[a]: Standard deviation.

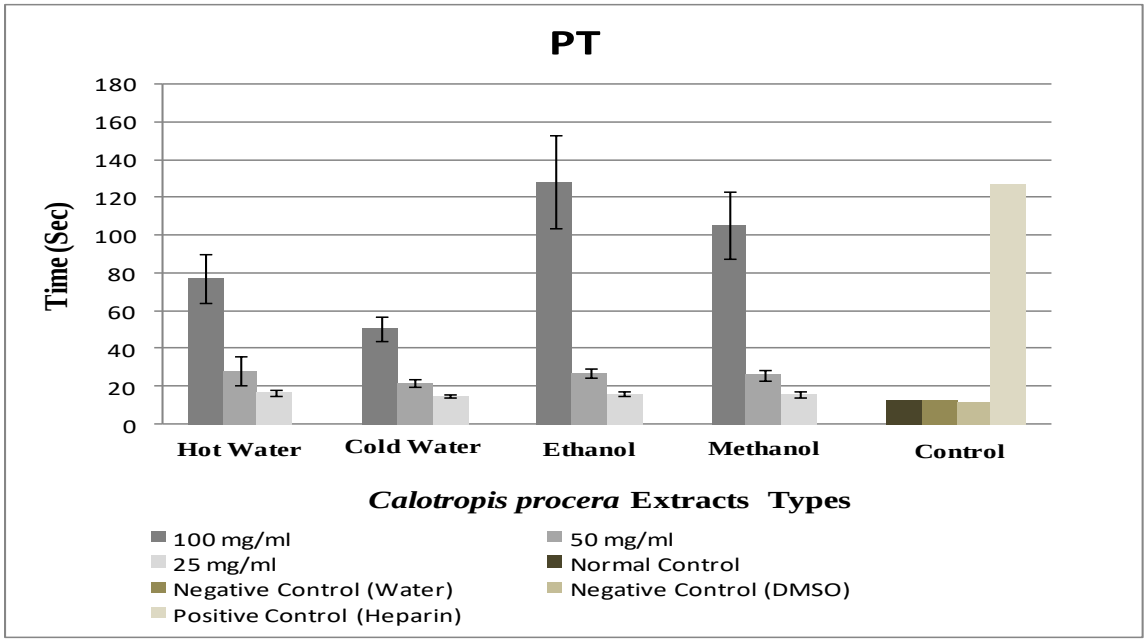

Figure (1): The PT values of the studied *Calotropis procera* extracts at different evaluated concentrations (100, 50, 25 mg/ml).

Nevertheless, all *Calotropis procera* different evaluated extract types significantly prolonged aPTT ($P \leq 0.05$) at concentrations100 and 50 mg/ml. The recorded anticoagulation activity at a concentration of 100 mg/ ml (420.00 ±0.00 sec) is considered to be remarkable compared to the normal control (25.21±1.75 sec). In contrast, all evaluated extract types at a concentration of 25 mg/ml showed the opposite effect: decreasing aPTT up to a point causing immediate blood clotting, zero seconds ($P \leq 0.05$).

Moreover, all evaluated plant extract types at 100 mg/ml concentrations revealed similar anticoagulation bioactivity on the studied blood samples aPTT compared to the positive heparin control ($P > 0.05$). As a result, they indicated their highest anticoagulation effect compared to other plant extract concentrations (50 and 25 mg/ml). The aPTT recorded results of all evaluated extract types at all investigated concentrations are represented in (Table 2 and Figure 2).

Table (2): The aPTT values of the studied *Calotropis procera* extracts at 100, 50, and 25 mg/ml concentrations.

Plant extracts	Concentration (mg/ml)	aPTT±SD[a] (sec)	* *P*-value	** *P*-value
Hot water	100	420.00±0.00	0.000	0.85
	50	51.70±27.06	0.000	0.000
	25	0.00±0.00***	------	------
Cold water	100	420.00±0.00	0.000	0.85
	50	129.97±56.66	0.000	0.000
	25	0.00±0.00***	------	-----
Ethanol	100	420.00±0.00	0.000	0.85
	50	86.70±28.85	0.000	0.000
	25	0.00±0.00***	------	------
Methanol	100	420.00±0.00	0.000	0.85
	50	79.97±16.39	0.000	0.000
	25	0.00±0.00***	------	------

Plant extracts	Concentration (mg/ml)	aPTT±SD[a] (sec)	* *P*-value	** *P*-value
Normal control (blood sample without plant extract)		25.21±1.75		
Negative control (SDW)		34.55±3.89		
Negative control (1%DMSO)		29.20±2.66		
Positive control (Heparin = 0.016 mg/ml)		420.00±0.00		

*P-value ≤0.05 is significant [between the different extract types investigated concentrations relative to the normal control].

**P-value ≤0.05 is significant [between the different extract types investigated concentrations relative to the positive control (heparin = 0.016 mg/ml)].

*** Coagulant effect of the plant extracts at that concentration, zero aPTT.

SD[a]: Standard deviation.

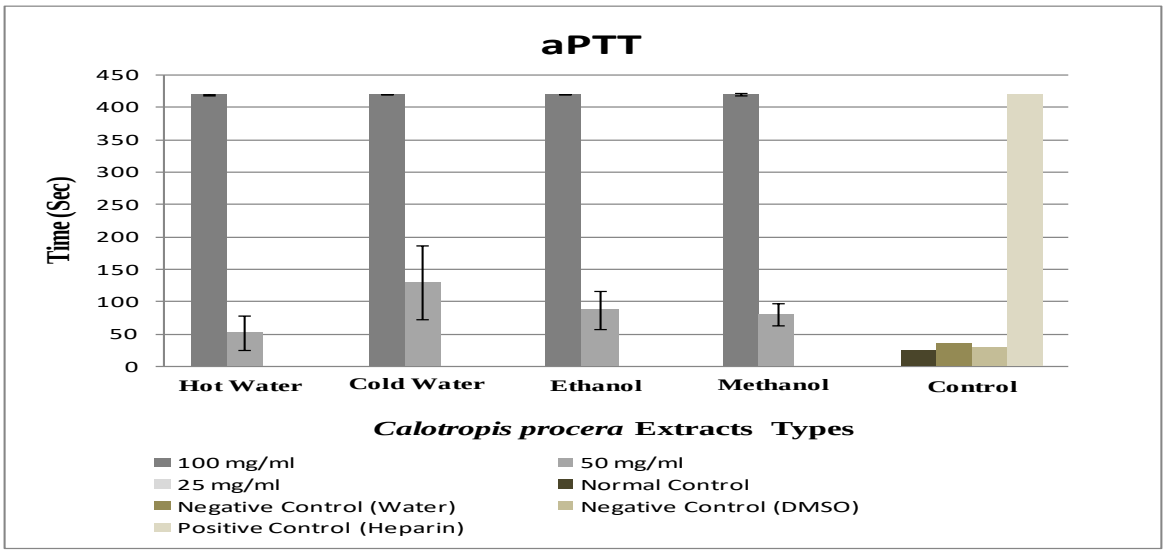

Figure (2): The aPTT values of the studied *Calotropis procera* extracts at different evaluated concentrations (100, 50, 25 mg/ml).

Moreover, the results showed that all extract types at concentrations of 100 and 50 mg/ml increased both PT and aPTT significantly (P≤0.05).

DISCUSSION

Blood coagulation is an important part of hemostasis [23]. The indicators of blood coagulation are PT and aPTT [2]. The PT is used to evaluate the coagulation factors V, VII, and X in the extrinsic pathway, while aPTT is used to evaluate the coagulation factors such as VIII, IX, XI, XII, and prekallikrein in the intrinsic coagulation pathway of the coagulation cascade. The normal value of PT is between 12.5 and 13.7 seconds and between 31 and 39 seconds for aPTT [18].

In this study, the results of PT and aPTT *in vitro* assays showed that *C. procera* leaf extracts had had an anticoagulation or coagulation effect on the examined blood samples. The obtained significant effect of the different extract types could be referred to as the plant extracts' constituents.

Since the evaluated plant extract types have prolonged PT, it could be suggested that they may have an inhibitory effect on the clotting factors that belong to the coagulation cascade's extrinsic (tissue factor) pathway [2, 24]. Furthermore, the four evaluated extract types exhibited a great potency of prolonging aPTT at 100 and 50 mg/ml concentrations. This finding suggests that the anticoagulant activity could be referred to as the inhibition of one or more clotting factors that belong to the intrinsic (contact factors) pathway [25] and/ or common pathway of the coagulation cascade [26].

On the contrary, these extracts at a 25 mg/ml concentration demonstrated a marked decreasing effect on the aPTT. They caused direct, immediate clotting of the examined blood samples recording zero aPTT. This out finding suggests that the plant could have accelerated the coagulation cascade by activating several clotting factors of the intrinsic pathway [9]. Also, the observed coagulation activity could be referred to as the proteolytic action on pure human fibrinogen [27]. Such discrepancies may be due to the total enzymes within the extracts [20] and the enzyme/enzymes' ability to hydrolyze fibrinogen and its subsequent polymerization to form fibrin threads [28]. This result complies with previous literature in that the latex of C. procera has a procoagulant effect, exhibits thrombin and plasmin-like activity, and reduces clotting time. Therefore, *C. procera* different evaluated extract types at a concentration of 25mg/ml may exhibit thrombin-like activity. As a result, they could be considered potential tools for activating the coagulation cascade, recommending their use under adverse physiological conditions such as bleeding, hemorrhagic, dengue, or hemophilia [20].

All evaluated extracts of *C. procera* prolonged PT and aPTT for concentrations of 100 and 50 mg/ml relative to the normal control. This indicates that these plant leaf extracts may have an inhibitory effect on the clotting factors in the intrinsic and extrinsic pathways and on the common pathway coagulation factors (X, V, and II) and fibrinogen [18, 25]. Also, this result may refer to a simple degradation (proteolytic action) of the proteins involved in the coagulation cascade [26].

Overall, the recorded results coincide with the literature in that various phytochemicals attenuate the coagulation cascade by inhibiting or decreasing the activity of tissue factors or thrombin [9]. In addition, the intrinsic and extrinsic pathways are activated or inhibited by biological substances present in some plants [4].

Because of that, previous literature has proved that the latex of C. *procera* has procoagulant activity; the results presented here could give the first scientific evidence for the widespread use of this plant leaves in complementary and alternative medicine for the treatment or prevention of coagulation disorders (thrombosis and hemorrhage).

These current research findings go along with the phytochemical screening of *C. procera* leaves, which proved the presence of sterols, triterpenes, flavonoids, cardiac glycosides, tannins, saponins, and alkaloids [29-31].

The presence of such phytochemicals in the plant species under study may explain their effect on the attenuation of the coagulation cascade. As phenol produced a definite, though suboptimal platelet factor 3 activity, evolved factor XII, accelerated thrombin–fibrinogen interaction, retarded clot retraction, enhanced the action of streptokinase on plasminogen and inhibited plasmin. In addition, it accelerated stypven clotting time and thrombin–fibrinogen reaction [32]. Furthermore, *in vitro* platelets aggregation inhibition was referred to as terpenoids which are phenols, explaining their extension tendencies to PT and aPPT [33].

Also, the effects of the well-known plant polyphenolic compounds on factor Xa revealed that polyphenols belonging to the flavonoids group: procyanidin B2, cyanidin, quercetin, and silybin, had an inhibitory effect on FXa activity. This indicates that flavonoids might be potential structural bases for the design of new nature-based, safe, orally bioavailable direct FXa inhibitors [34]. Moreover, the flavonoids and tannins proved their effect on the PT and aPPT [35], which went along with a hemostatic mechanism *in vivo* study that previously proved flavonoids' effect on the coagulation cascade [36].

Furthermore, plants are known for their carbohydrates and proteins, which were recorded to have bioactivity on blood hemostasis. For example, polysaccharides such as pectins and hemicelluloses isolated from different plant species demonstrated antithrombin and thrombolytic activity [4]. Similarly, plant lectins affect blood hemostasis by increasing PT and aPTT [37]. In addition, cysteine proteases extracted from several plants' latex exhibit fibrinolytic activity and reduce clotting time [13, 20, 38].

As a result, plant extracts contain variable constituents which show different hemostatic bioactivity based on the type of extraction of a particular plant species. Therefore, this fact is confirmed by the bioactivities of different plant species can be attributed to a synergic action among the molecules [39]. So, the

efficacy of the plant species extracts evaluated as anticoagulants or coagulants was related to various degrees of solubility of different molecules [40].

CONCLUSION

This study can be considered the first of its kind on *C. procera* anticoagulant and coagulant effect in Palestine. However, despite those fundamental recorded results of this research that all evaluated extracts possess potent anticoagulant and procoagulant activity *in vitro*, it is not conclusive as *in vivo*. Consequently, further investigations are recommended considering *in vivo* anticoagulant bioactivity of *C. procera in* different plant extracts. In addition, further mutual physiological and cytotoxicity detection could be considered a must for more clarity. It is noteworthy that the use of this plant's herbal preparations before undergoing any surgical procedure should be ceased as a safety precaution, based on their obtained anticoagulant and coagulant effects [9].

Furthermore, from the out findings in this study, it is proposed to identify anticoagulant and coagulant potent molecules and their biological characterization to be exploited as a resource for new natural agents and identify the most affected specific clotting factors in the coagulation cascade is recommended. Moreover, studying the histopathological effects of *C. procera* extracts could be an important field of research. Also, a comparative study between the latex and leaf extracts of *C. procera* could be investigated to reveal the synergistic or antagonist effect among the different plant species extracts types.

Consent for publication

We declare that all of the authors have read and approved the paper. The paper has not been published previously, nor is it considered by any other journal. The authors give the Publisher the Author's permission to publish the work.

Data Availability

All data generated for this study are included in the article.

Author Contributions

Ghadeer Omar: conceptualization, writing-original draft, formal analysis, methodology, project administration, supervision, validation, visualization, and writing review & editing. **Kholoud Thiab:** data curation, formal analysis, investigation, resources. **Ghaleb Adwan:** conceptualization, writing-original draft, formal analysis, methodology, project administration, supervision, validation, visualization, and writing review & editing. **Ali Barakat:** statistical analysis.

This research was carried out by Kholoud Thiab (a master's student, An-Najah National University, Department of Biology and Biotechnology).

Competing Interest

The authors declare that there are no conflicts of interest.

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Ethics approval and consent to participate

Ethics approval was obtained from the Institutional Review Board of An-Najah National University.

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