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In vitro anticoagulant effect of methanolic extracts of *Artemisia herba-alba*, *Achillea fragrantissima* and *Citrullus colocynthis* grown in Saudi Arabia

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Several medicinal plants are known to possess anticoagulant activity. This study proposed to evaluate the *in vitro* anticoagulant effect of methanol extracts of *Citrullus colocynthis* (fruits), *Achillea fragrantissima* (aerial parts) and *Artemisia herba-alba* (aerial parts) on normal human plasma. A total of 100 blood samples used in this study were taken from healthy volunteers at the Hematology laboratory of King Khalid Hospital, Al-Majmaah, Kingdom of Saudi Arabia from November 2018 to March 2019, using 3.2% sodium citrate tubes with a collection ratio of 9:1 blood to anticoagulant. PT, INR and PTT were used to assess the anticoagulant activity of these plants. The highest PT results were observed for 1000 µg/mL methanol extract of *C. colocynthis* (33.7±3.4 s) followed by 1000 µg/mL methanol extract of *A. fragrantissima* (28.2±2.6 s) and 1000 µg/mL methanol extract of *A. herba-alba* (26.4±0.2 s). Moreover, the highest PTT values were observed for 250, 500 and 1000 µg/mL *A. fragrantissima* (87.9±1.0, 97.9±5.1 and 112.5±1.1 s, respectively), 500 µg/mL and 1000 µg/mL *C. colocynthis* (65.1±1.0 and 106.4±0.4 s, respectively) and 1000 µg/mL *A. herba-alba* (157.0±3.0 s). The anticoagulant effect of methanol extracts of *A. herba-alba*, *A. fragrantissima*, and *C. colocynthis* were evaluated using coagulation assays, in which prolonged clotting time of their methanol extracts was observed. These findings potentially justify the use of these plants in managing arterial and venous thrombotic disorders.

Keywords: *Artemisia herba-alba*, *Achillea fragrantissima*, *Citrullus colocynthis*, International normalized ratio, Prothrombin time, Partial thromboplastin time

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Anticoagulant drugs such as heparin have been the backbone of the treatment of arterial and venous thrombotic conditions for decades, but they have some complications. Previous studies have indicated that a few medicinal plants are known for their anticoagulant activity, indicating a novel therapeutic approach to treating arterial and venous thrombotic disorders[1-4].

Artemisia herba-alba (Asteraceae) is a greyish strongly aromatic dwarf shrub, (Arabic name: chih[5]). *A. herba-alba* has broad range of uses in folk medicine, namely, treatment of diarrhea and abdominal cramps, external wound healing,[6,7] treatment of helminthiasis, diabetes mellitus and jaundice[8]. The pharmacological properties of *A. herba-alba* extract include antidiabetic,[9-12] antimicrobial,[13] antioxidant[14,15] and antisickling activities[16].

Achillea fragrantissima (Asteraceae) is a wild herbaceous shrub[17] that has several different common names depending on the country they are found in, such as Lavender cotton (English), Guarda roba (French) and Qaysūm (Arabic)[18]. It has been used in traditional medicine for the treatment of respiratory, skin and gastrointestinal diseases, hypertension, and diabetes[19-21]. Several reports demonstrated different pharmacological activities of *A. fragrantissima* extracts[22-24].

A. fragrantissima lacks anti-rheumatic or anti-inflammatory effects in carrageenan-induced acute inflammation in rats[25] but has antimicrobial and antiviral activities[26,27]. Its modulatory effects were observed on rat ileum muscle contraction[28], and it is therefore useful in avoiding neurodegenerative diseases[23]. Aqueous extract of this herb exhibited strong cytotoxicity and larvicidal activities[29,30]. Antioxidant, antimicrobial, antiplatelet, anti-proliferative and acetylcholinesterase (AChE) inhibition efficacy of aqueous and hydro-alcoholic extracts from *A. fragrantissima* grown in Jordan were studied[31].

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Citrullus colocynthis (Cucurbitaceae) is a medicinal plant that has several different common names, such as colocynth, bitter apple, wild gourd and the Arabic names Handal and Hhanzal. The fruit of *C. colocynthis* contains a bitter glycoside, which has ample medicinal values. Aqueous and methanol extracts of *C. colocynthis* showed high antimicrobial activity as well as anticancer, proapoptotic, and antidiabetic properties[32-35].

Several studies reported anticoagulant effects of plant extracts and their chemical constituents[1-4,36,37]. The reason of selecting these three plant species to test their anticoagulant activity is because there are no published studies displaying the *in vitro* anticoagulant effect of these three plant species. Therefore, this study was proposed to evaluate the *in vitro* anticoagulant effect of methanol extracts of *A. herba-alba*, *A. fragrantissima* and *C. colocynthis* on normal human plasma.

In vitro anticoagulant effect of methanol extracts of *A. herba-alba* Asso, *A. fragrantissima*, and *C. colocynthis* was evaluated by measuring prothrombin time (PT), international normalized ratio (INR) and partial thromboplastin time (PTT). These were determined for plasma samples using the coagulation analyzer.

Methodology

Procedure for the formation of plant methanol extracts

In our current research, *A. fragrantissima* (aerial parts), *A. herba-alba* (aerial parts) and *C. colocynthis* (fruits) were collected from Al Majmaah Riyadh region, Saudi Arabia in December 2017. All plant parts were dried under the same conditions. Dried plant samples were ground in the same blender for preparing identical size of the powder. Thereafter, 100 g of powder was added in 1 L of 80% CH_3OH (methanol) for 1 week at room temperature. A wide range of phyto-chemical compounds both polar and non-polar are brought out by methanol easily because methanol contains both polar and non polar groups which make it able to extract both polar and non polar compounds. Another reason is the low boiling point of methanol. It helps to evaporate the filtrate within a short time. Moreover, methanol is less carcinogenic than other solvents. Also the market price is less than other solvents.

The mixture was filtered and the filtrate was vaporized with the help of rotary evaporator for removal of CH_3OH at 50°C under reduced pressure. Now we get the final dry crude extract in the dark color glass bottle for further use in research study at refrigerator. 0.1 g/mL (stock solution) of crude extract was formed by dissolving 0.1 g of dry crude extract in 1 mL dimethylsulfoxide (DMSO) and we further diluted in 9 mL 0.9% NaCl. For 5 days, this stock solution was kept in refrigerator.

Collection of blood samples

Properly collected specimen leads to good blood test result, so blood sample should be collected into a tube with oxalate or citrate ions to remove the calcium from plasma sample to prevent the clotting process from beginning before the test (Prothrombin Time test and partial thromboplastin time test) and that's because PT and PTT test check if blood clotting factors are present.

A total of 100 blood samples were taken from healthy volunteers at the Hematology Laboratory of King Khalid Hospital, Al-Majmaah, Kingdom of Saudi Arabia from November 2018 to March 2019. Informed consent was obtained from each study subject prior to their participation in the research. The blood samples were collected using 3.2% sodium citrate tubes with a collection ratio of 9:1 blood to anticoagulant. These sodium citrate tubes were filled completely. Samples were gently mixed after collection and stored for a maximum of 1 h. All procedures were in accordance to ethical standards of the Ethical Committee, King Fahad Medical City, Ministry of Health, Kingdom of Saudi Arabia, approval number: IRB-KFMC: Log. Number: 18-570E

Anticoagulant activity

Preparation of platelet poor plasma (PPP)

Sodium citrate tubes were centrifuged to get PPP by centrifugation at 2000 ×*g* for 15 min to separate plasma. The plasma samples were stored at 4°C until required for further use.

Anticoagulant activity evaluation

In order to evaluate the anticoagulant effect of plant extracts, *in vitro* anticoagulant assay was performed as follows:

To evaluate the extrinsic pathway of coagulation, PT and INR tests were performed, using normal human plasma. To evaluate the intrinsic pathway of coagulation, PTT test was done, using normal human plasma.

PT test

The extrinsic pathway was assessed using the PT test, with some modifications[38]. Plasma (500 µL) was mixed with 500 µL of different concentrations

(250, 500, 1000 and 10000 µg/mL) of crude extracts and incubated for 60 min at 37°C. Thereafter, the pre incubated plasma was used for determination the PT test and this was carried out using a coagulation analyzer (Sysmex CA-1500) in the hematology lab at King Khalid Hospital in AL-Majmaah. 0.9% NaCl was used as the negative control.

INR test

The extrinsic pathway was also evaluated using the INR test, with some modifications[38]. Plasma (500 µL was mixed with 500 µL of different concentrations (250, 500, 1000 and 10000 µg/mL) of crude extracts and incubated for 60 min at 37°C. Thereafter, the pre incubated plasma samples were used for determination the INR test and this was carried out using a coagulation analyzer (Sysmex CA-1500) in the hematology lab at King Khalid Hospital in AL-Majmaah. 0.9% NaCl was used as the negative control.

PTT test

The action in intrinsic and common pathways was assessed using the PTT test, with some modifications[38]. Plasma (500 µL) was mixed with 500 µL of different concentrations (250, 500, 1000 and 10000 µg/mL) of crude extracts and incubated for 60 min at 37°C. Thereafter, the preincubated plasma was used for determination via the PTT test and this was carried out using a coagulation analyzer (Sysmex CA-1500) in the hematology lab at King Khalid Hospital in AL-Majmaah. NaCl (0.9%) was used as the negative control.

A stock solution (10 µg/mL) of plant extract was formed by suspending 0.1 g of dry extract of each plant in 1 mL of 100% DMSO, prior diluted to 10 mL with normal saline. Then we formed 3 dilutions (250, 500 and 1000 µg/mL) from the stock solution of plant extracts.

Statistical analysis

Data were analyzed by using SPSS (Statistical Package for Social Sciences) software, version.17.

Results

Extractive yield

The yields (g extract/100 g dry plant part) of studied plants are shown in Table 1. The extraction yield varied from the highest yield of 19.0% in *A. herba-alba* to the lowest yield of 5.0% in *C. colocynthis*.

Table 1 — Percentage (%) yield (g extract/100 g dry plant part) of studied plant species

Plant species	% Yield
Achillea fragrantissima (Forssk) Sch. Bip (aerial parts)	12.0
Artemisia herba-alba Asso (aerial parts)	19.0
Citrullus colocynthis (L.) Schrad (fruits)	5.0

Anticoagulant activity of methanol extracts of studied plants

The anticoagulant activity of plant extracts was evaluated by PT, INR and PTT assays, using normal human plasma.

Effect of methanol extracts of studied plants on PT

To evaluate the extrinsic pathway of coagulation, the PT assay was done, using normal human plasma.

Table 2 shows PT results after incubation of normal human plasma with 0.9% NaCl (control) and with 250, 500 and 1000 µg/mL of crude extracts of *A. herba-alba* (aerial parts), *A. fragrantissima* (aerial parts) and *C. colocynthis* (fruits).

As shown in Table 2, the highest PT result was observed for 1000 µg/mL of methanolic extract of *C. colocynthis* (fruits) (33.7±3.4 s) followed by 1000 µg/mL of methanol extract of *A. fragrantissima* (aerial parts) (28.2±2.6 s) and 1000 µg/mL of methanol extract of *A. herba-alba (*aerial parts) (26.4±0.2 s). Moreover, a significant increase in PT results, compared to the control, was observed at concentrations of 500 and 1000 µg/mL of methanol extracts of *A. fragrantissima* and *A. herba-alba*. These findings indicate the significant anticoagulant effect of these plants. Significant increase in PT results was also observed at 250, 500 and 1000 µg/mL of methanol extracts of *C. colocynthis*, which also proved its anticoagulant activity.

Effect of methanol extract of studied plants on PTT of normal human plasma

To evaluate the intrinsic pathway of coagulation, the PTT assay was done, using normal human plasma.

Table 3 shows PTT results after incubation of normal human plasma with 0.9% NaCl (control), and with 250, 500 and 1000 µg/mL of crude extracts of *A. herba-alba* (aerial parts), *A. fragrantissima* (aerial parts) and *C. colocynthis* (fruits).

As shown in Table 3, the highest PTT values were observed at 250, 500, and 1000 µg/mL concentrations of methanol extract of *A. fragrantissima* (87.9±1.0, 97.9±5.1 and 112.5±1.1 s, respectively). High PTT values were also observed at 500 and 1000 µg/mL concentrations of methanol extract of *C. colocynthis* (65.1±1.0 and 106.4±0.4 s, respectively) and at

Table 2 — Effect of methanol extract of studied plants on Prothrombin Time (PT) of normal human plasma

Plant Species	Prothrombin Time (PT) in seconds			
	Plasma + 250 µg/mL	Plasma + 500 µg/mL	Plasma + 1000 µg/mL	Plasma + normal saline (Control)
Achillea fragrantissima	13.3 ± 1.0	20.1 ± 1.7	28.2 ± 2.6	13.7±0.9
P value	0.5830	0.0047	0.0008	
Artemisia herba-alba	13.6 ± 1.4	15.3 ± 1.6	26.4 ± 0.2	11.5 ± 0.2
P value	0.0668	0.0154	0.0001	
Citrullus colocynthis	14.1 ± 0.6	16.5 ± 0.3	33.7 ± 3.4	11.7 ± 0.5
P value	0.0076	0.0002	0.0004	

Each value represents the mean value ± S.D., (n =3), p value ≤0.05 was considered significant, compared to the control.

Table 3 — Effect of methanol extract of studied plants on Partial Thromboplastin Time (PTT) of normal human plasma

Plant Species	Partial Thromboplastin Time (PTT) in seconds			
	Plasma + 250 µg/mL	Plasma + 500 µg/mL	Plasma +1000 µg/mL	Plasma + normal saline (control)
Achillea fragrantissima	87.9 ± 1.0	97.9 ± 5.1	112.5 ± 1.1	58.1 ± 1.6
P value	0.0001	0.0002	0.0001	
Artemisia herba-alba	56.0 ± 0.4	56.9 ± 1.0	157.0 ± 3.0	58.1 ± 1.6
P value	0.0962	0.3392	0.0001	
Citrullus colocynthis	NM	65.1 ± 1.0	106.4 ± 0.4	52.1 ± 0.8
P value	ND	0.0001	0.0001	

Each value represents the mean value ± S.D., (n =3), p value ≤0.05 was considered significant, compared to the control. NM indicates not measured.

Table 4 — Effect of methanol extract of studied plants on International Normalized Ratio (INR) of normal human plasma

Plant Species	International Normalized Ratio (INR) in seconds			
	Plasma + 250 µg/mL	Plasma + 500 µg/mL	Plasma + 1000 µg/mL	Plasma + normal saline (control)
Achillea fragrantissima	1.2 ± 0.2	1.8 ± 0.1	2.5 ± 0.2	1.2 ± 0.1
P value	0.5857	0.0047	0.0008	
Artemisia herba-alba	1.2 ± 0.1	1.3 ± 0.1	2.3 ± 0.01	1.0 ± 0.01
P value	0.0649	0.0142	0.0001	
Citrullus colocynthis	1.2 ± 0.05	1.5 ± 0.006	2.9 ± 0.3	1.04 ± 0.04
P value	0.0069	0.0001	0.0004	

Each value represents the mean value ± S.D., (n =3), p value ≤0.05 was considered significant, compared to the control.

1000 µg/mL concentration of methanol extract of *A. herba-alba* (157.0±3.0 s).

Effect of methanol extracts of studied plants on INR

Table 4 shows INR values after incubation of normal human plasma with 0.9% NaCl (control) and with 250, 500 and 1000 µg/mL of crude extract of *A. herba-alba* (aerial parts), *A. fragrantissima* (aerial parts) and *C. colocynthis* (fruits).

As shown in Table 4, the highest INR value was observed for 1000 µg/mL of methanol extract of *C. colocynthis* (2.9±0.3) followed by 1000 µg/mL of methanol extract of *A. fragrantissima* (2.5±0.2) and by 1000 µg/mL of methanol extract of *A. herba-alba* (2.3±0.01).

A significant increase in INR results, compared to the control, was observed at concentrations of 500 and 1000 µg/mL of methanol extracts of *A. fragrantissima*, *A. herba-alba* and *C. colocynthis*. These findings indicated significant anticoagulant activity of methanol extracts of the studied plants.

Discussion

Previous studies reported that medicinal plants possess anticoagulant activity, contributing to the treatment of arterial and venous thrombotic disorders. The present study evaluates the *in vitro* anticoagulant effect of *C. colocynthis* (fruits), *A. fragrantissima* (aerial parts) and *A. herba-alba* (aerial parts)

methanol extracts. The anticoagulant activity of plant extracts was evaluated by the PT, INR and PTT assays, using normal human plasma.

After incubation of normal human plasma with 0.9% NaCl (control), and with 250, 500 and 1000 µg/mL of crude extract of *A. herba-alba* (aerial parts), *A. fragrantissima* (aerial parts) and *C. colocynthis* (fruits), the highest PT and INR results were observed for 1000 µg/mL of methanol extract of *C. colocynthis* (fruits) (33.7±3.4 s, 2.9±0.3) followed by 1000 µg/mL of methanol extract of *A. fragrantissima* (aerial parts) (28.2±2.6 s, 2.5±0.2) and by 1000 µg/mL of methanol extract of *A. herba-alba* (aerial parts) (26.4±0.2 s, 2.3±0.01). These results indicate that the methanol extracts of studied plants prolonged clotting time as recorded via the PT test and this prolongation of PT indicates the inhibition of the extrinsic and/or common pathway of coagulation, demonstrating their anticoagulant activity.

These findings coincide with the findings of previous studies[1-4,36,37]. A significant increase in PT results, compared to the control, was observed at concentrations of 500 and 1000 µg/mL of methanol extracts of *A. fragrantissima* and *A. herba-alba*. These findings indicate the significant anticoagulant activity of these plants. A significant increase in PT results was also observed at 250, 500 and 1000 µg/mL of methanol extracts of *C. colocynthis*, also proving its anticoagulant activity.

After incubation of normal human plasma with 0.9% NaCl (control) and with 250, 500 and 1000 µg/mL of crude extract of *A. herba-alba* (aerial parts), *A. fragrantissima* (aerial parts) and *C. colocynthis* (fruits), the highest PTT values were observed at 250, 500 and 1000 µg/mL concentrations of methanol extracts of *A. fragrantissima* (87.9±1.0, 97.9±5.1 and 112.5±1.1 s, respectively). The methanol extracts of *A. fragrantissima* were able to prolong the clotting time observed via the PTT test, indicating its anticoagulant activity. High PTT values were also observed at 500 and 1000 µg/mL concentrations of methanolic extracts of *C. colocynthis* (65.1±1.0 and 106.4±0.4 s, respectively). These findings indicated significant anticoagulant activity of *C. colocynthis*. High PTT values were observed at 1000 µg/mL concentration of methanol extracts of *A. herba-alba* (157.0±3.0 s). The methanol extracts of *A. herba-alba* were able to prolong the clotting time in the PTT test only at high concentration, indicating its anticoagulant activity.

The methanol extracts of studied plants were able to prolong the clotting time in the PTT test. This prolongation of PTT indicates the inhibition of the intrinsic and/or common pathway of coagulation demonstrating their anticoagulant activity. These findings coincide with the findings of a previous study[4,36,37].

Our findings in the current study revealed that the three methanol extracts of studied plants showed anticoagulant activity via inhibition of coagulation pathways. Further studies are recommended to identify the active constituents in each plant extract that may be responsible for the anticoagulant activity of these three plants.

Conclusion

The present study demonstrated that methanol (aerial parts, fruits) extract of *A. herba-alba*, *A. fragrantissima,* and *C. colocynthis* possesses significant *in vitro* anticoagulant activity. These findings potentially justified the use of this plant in the management of arterial and venous thrombotic disorders.

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Conflict of interest

Authors have declared that there are no conflicts of interest.

Authors' contributions

N A and A A designed the study and performed data collection, processing and analysis. All authors wrote and approved the final version of the manuscript.

Ethical consent

This study was approved by the Ethical Committee, King Fahad Medical City, Ministry of Health, Kingdom of Saudi Arabia, approval number: IRB-KFMC: Log. Number: 18-570E.

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