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Original Research Article

Selected Wild Plants Ethanol Extracts Bioactivity On The Coagulation Cascade

ABSTRACT

Aim: As there is little data available on the validity of wild plants use in Palestine for blood disorders, the aim of this study was to determine the anticoagulant properties of *Urtica urens*, *Parietaria judica*, *Satureja thymbra*, *Thymbra spicata, Teucrium creticum*, *Verbascum fruticulosum*, *Lupinus pilosus*, *Paronychia argentea*, and *Ruta chalepensis*.
Place and Duration of Study: Department of Biotechnology and Biology/ Faculty of Science/ An-Najah National University, between November 2015 and May 2016.
Methodology: Studied plant species ethanol extracts were prepared to final concentrations 12.5, 25 and 50 mg/ml. In vitro PT and aPTT assays were conducted on normal platelet poor plasma blood samples by a digital coagulation analyzer. Statistical analysis of the results conducted using a statistical package SPSS via applying mean values using one-way ANOVA with post-hoc tests.
Results: *Urtica urens* extract prolonged PT at 50 mg/ml, while *T. spicata* at 50 and 25 mg/ml, suggesting their inhibitory effect on the tissue clotting factors, which belong to the extrinsic pathway of the coagulation cascade. *Paronychia argentea* demonstrated a decreasing effect on PT at all studied concentrations recording zero PT, affecting the extrinsic pathway. Furthermore, *U. urens*, *T. spicata*, *P. argentea* and *P. judica* prolonged aPTT at 50 mg/ml due to the inhibition of the contact factors in the intrinsic pathway. The greatest anticoagulation activity was seen in *U. urens* and *T. spicata* as they prolonged both PT and aPTT, so they could have inhibitory effect not only on the clotting factors in the intrinsic and extrinsic pathways, but also, those in the common pathways.
Conclusion: the effective examined plant species provide potential bioactivity from which anticoagulation or anti-bleeding drugs can be exploited.

Keywords: aPTT; PT; Medicinal plants; Coagulation cascade

1. INTRODUCTION

Haemostasis is very in vital under physiological conditions in which the processes of clot formation, anticoagulation and clot dissolution are all in balance. However, the control of such process is very crucial as inappropriate blood clotting is responsible for a large number of deaths [1]. Blood-clotting (coagulation) involves platelets, enzymes and clotting factors among of which the first ones, are regarded as key regulators of both haemostasis and pathogenesis of cardiovascular diseases [2, 3]. On one hand, finding effective treatment for the atherothrombotic diseases, strokes and other cardiovascular diseases is a hot spot of concern [3]. On the other hand, the clinical limitations and adverse side effects associated with the currently used anti-thrombotic agents have fuelled the search for new, safer and effective anti-thrombotic aggregation agents of natural origin. For example, the widely used heparin anticoagulant therapy induces severe side effects over the long term [4, 5]. In addition to that the rising costs of prescription drugs in the maintenance of personal health have increased the interest in medicinal plants. Medicinal plants are rich sources of biologically active compounds [6], which are commonly found at varying concentrations in one or more different parts of a plant. They are either individually or synergistically responsible for the various therapeutic properties of medicinal plants [7]. Therefore, in recent years, naturally occurring chemical substances derived from plants have attracted interest as possible treatments for coagulation disorders and as template molecules for the development of new drugs [8].
The World Health Organization (WHO) estimates that 65-80% of the world's population use traditional medicine as their primary form of health care [9, 10]. While stressing the dangers of using traditional

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medicines, it should not be forgotten that a multitude of life-saving drugs have been obtained from plants. As the herbal drugs are wide-spoken as green medicine for their safe and dependable health care paradigms, tremendous efforts have been directed towards the discovery and development of natural products with antiplatelet [11,12], anticoagulant [13,14] and antithrombotic [15] activity of the plants. Ethnobotanical survey revealed that various medicinal plants stood out to manage blood-clotting related diseases [16-18]. Moreover, epidemiologic studies have provided evidence that foods with experimentally proved antithrombotic effect could reduce risk of thrombosis. Some plants or plant parts showing thrombolytic activity have been reported [19, 20]. A recent high demand in both developed and developing countries on natural products and their derivatives has resulted in an upsurge interest in medicinal plants as an alternative medicine [21].

Several studies in Palestine have been published concerning many plant extracts biological active properties such as antibacterial, antitumor, antifungal and antioxidant of wild plants [22]. As the intrinsic and extrinsic blood coagulation pathways can be detected using various in vitro assays, including prothrombin time, partial thromboplastin time and thrombin time [23]. A study considered the anticoagulation effect of the wild plants in Palestine was conducted on Viscum album extracts from olive and almond host plants. It showed significant prolongation effect on PT and aPTT [24].

As there is little data available on the validity of wild plants use in Palestine for blood disorders or wound healing, the aim of this study was to determine the anticoagulant properties of Urtica urens L., Parietaria judica L. (Urticaceae), Satureja thymbra L., Thymbra spicata L., Teucrium creticum L. (Lamiaceae), Verbascum fruticulosum Post (Scrophulariaceae), Lupinus pilosus L. (Fabaceae), Paronychia argentea Lam. (Caryophyllaceae), and Ruta chalepensis L. (Rutaceae). The anticoagulant activity in this study was performed directly on samples of human blood by determining the PT and the aPTT, which are one of the most important tests to monitor coagulation and anticoagulant effects.

2. MATERIAL AND METHODS

2.1. Plant Materials

The wild plant species (*Urtica urens* L., *Satureja thymbra* L., *Verbascum fruticulosum* Post*, Thymbra spicata* L.*, Lupinus pilosus* L., *Teucrium creticum* L., *Paronychia argentea* Lam., *Parietaria judica* L. and *Ruta chalepensis* L.) were collected from different locations in West Bank, Palestine. The collected plant species were identified by Ghadeer Omar, Department of Biology & Biotechnology, An-Najah National University; Palestine. Representative plant specimens of the nine plant species under investigation were collected, pressed till drying, treated chemically, mounted on herbarium sheets and provided with voucher numbers, and then they were deposited at An-Najah National University herbarium. The aerial parts of plant materials were washed, air dried, ground into powder using grinder and stored at room temperature until they were used.

2.2. Plant Extraction Procedure

Ten grams of each plant powder were soaked in 100 ml of 70% ethanol for one week at room temperature with interval shaking. Then the mixtures were centrifuged for 5 min at 5000 rpm. The supernatants were evaporated by a rotary evaporator. The obtained powder of each of the plant species was dissolved in 1% dimethyl sulfoxide (DMSO) to a final concentrations equal to 50, 25, 12.5 mg/ml.

2.3. Blood Sample Preparation

Five healthy volunteers were asked to give blood samples. The volunteers were not under any medication and not smokers. The citrated blood samples were prepared as the following; each blood sample was centrifuged at 3000 rpm for 15 min to obtain the Platelets Poor Plasma (PPP) [25]. All samples were subjected to PT and aPTT assays within 2 hours after blood collection. The clotting time for both tests was recorded by a digital coagulation analyzer (Coa DATA 4004, LAberBioMedical Technologies, Germany). All measurements were carried out in triplicate. The negative control for PT and aPTT assays was 1% DMSO.

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2.4. Prothrombin Time (PT) Assay

For *in vitro* PT assay, 50 µl normal citrated (PPP) was incubated with 50 µl from each plant extract at different concentrations (50, 25 and 12.5 mg/ml) for 5 min at 37℃. Clotting time was immediately recorded after the addition of 100 µl PT reagent (Hemostat thromboplastin-SI. Human, Germany).

2.5. Activated Partial Thromboplastin Time (Aptt) Assay

For *in vitro* aPTT assay, 50 µl normal citrated (PPP) was incubated with 50 µl from each plant extract at different concentrations (50, 25 and 12.5 mg/ml) for 2 min at 37℃. Then 50 µl aPTT reagent (Human, Germany) was added and incubated for further 3 min at 37℃. The aPTT clotting time was immediately recorded after the addition of 100 µl calcium chloride solution (Human, Germany).

2.6. Statistical Analysis

Statistical analysis of the PT and aPTT results was conducted using a statistical package SPSS via applying mean values using one-way ANOVA with post-hoc tests to determine if there was a significant difference among the different studied wild plant species and among the different concentrations of each examined plant species extract relative to the control. P value < 0.05 was considered to be significant.
Tables should be explanatory enough to be understandable without any text reference. Double spacing should be maintained throughout the table, including table headings and footnotes. Table headings should be placed above the table. Footnotes should be placed below the table with superscript lowercase letters.

3. RESULTS

The recorded PT values of the examined volunteers blood samples showed no individual variations ($p < .01$). As a result they would be considered as representative blood samples for this study. Their PT values were examined under the effect of different plant ethanol extracts at different concentrations (12.5, 25 and 50 mg/ml). Results revealed significant anticoagulation effect of some of the examined plant species at 50 and 25 mg/ml ($p = .05$). *Urtica urens* and *T. spicata* prolonged the PT significantly ($p = .05$) relative to the control at 50 mg/ml, while *T. spicata* has had anticoagulation effect at 25 mg/ml ($p = .05$). Their effect was concentration dependent ($p = .05$). However, this dose dependent behavior was also observed in *R. chalepensis* and *S. thymbra* ($p = .05$) in spite of the absence of significant anticoagulation effect of examined concentrations.
But *P. argentea*, showed an opposite effect by decreasing the PT up to a point causing immediate blood clotting at all examined concentrations.
The detailed PT results of all effective and non-effective studied plant species are represented in Table 1. and Figure 1.

Table 1. The effect of the studied plant species extracts at different concentrations (50, 25 & 12.5 mg/ml) on PT values of five human platelets poor plasma samples.

Plant Species	Concentration (mg/ml)	PT (s)	*P value	**P value
Lupinus pilosus	50	20.12	.571	.144
	25	15.3	.865	
	12.5	13.24	1	
Parietaria judaica	50	15.87	.985	.505
	25	12.9	1	

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	12.5	12.5	.878	
	50	0	.002	
Paronychia argentea	25	0	.002	.001
	12.5	0	.002	
	50	16.01	.982	
Ruta chalepensis	25	13.27	1	.035
	12.5	12.95	1	
	50	26.43	.065	
Satureja thymbra	25	17.24	.425	.001
	12.5	14.82	1	
	50	19.99	.589	
Teucrium creticum	25	14.7	.951	.097
	12.5	13.01	1	
	50	33.13	.002	
Thymbra spicata	25	22.24	.011	.034
	12.5	13.68	1	
	50	38.41	.001	
Urtica urens	25	17.1	.455	0.001
	12.5	13.89	1	
	50	22.5	.291	
Verbascum fruticulosum	25	16.3	.644	.609
	12.5	13.44	.203	

**p value = .05 was significant among the different studied plant species relative to the control (blood sample without plant extract). **p value = .05 was significant among the different concentrations of each examined plant species.*

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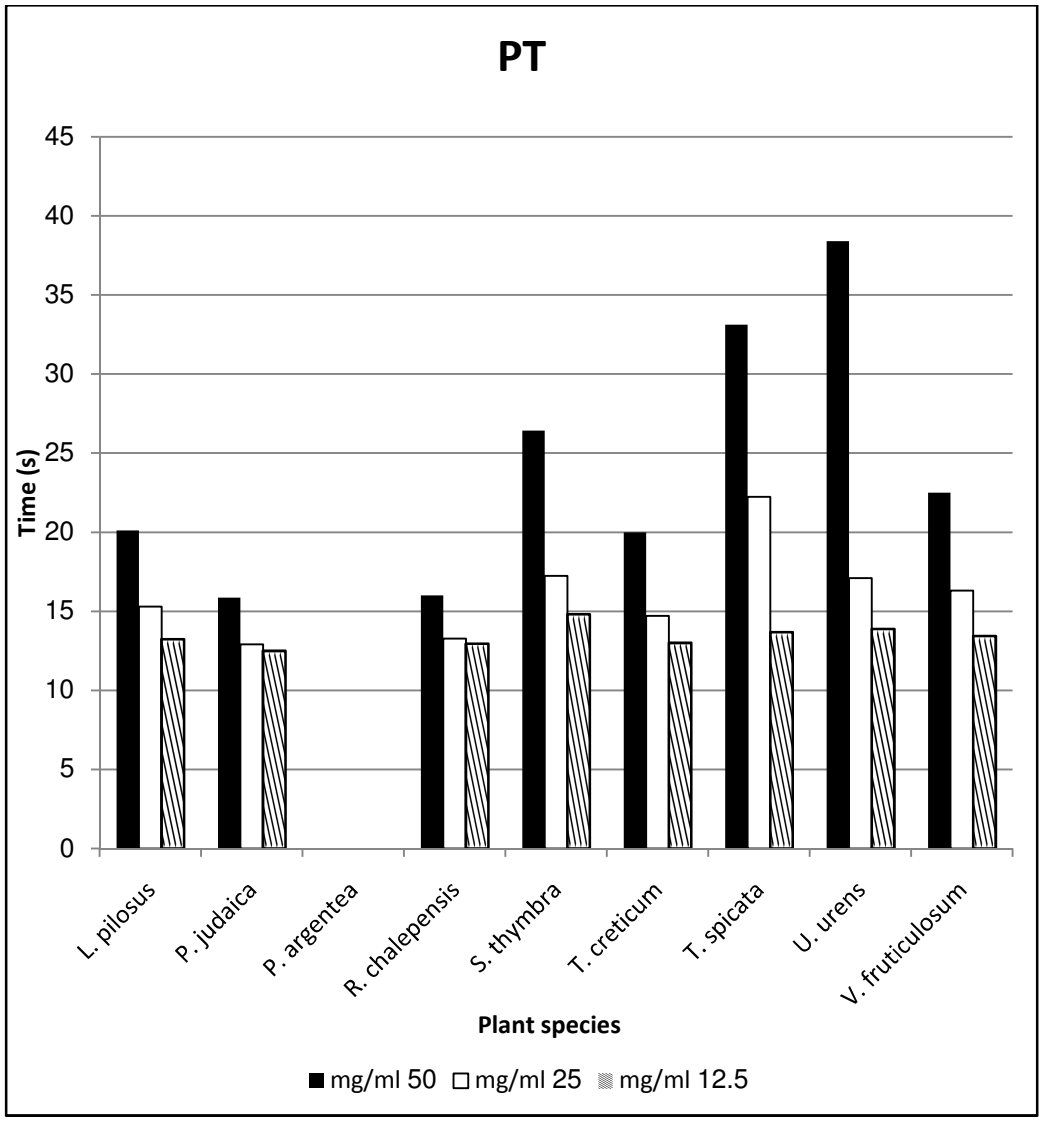

Fig. 1. The effect of the studied plant species extracts on PT values of five human platelets poor plasma samples at 50, 25 & 12.5 mg/ml concentrations.

Also the examined blood samples were considered as representative for the aPTT assay as they have had no individual variations in their recorded aPTT values (p =.998). *Urtica urens*, *T. spicata*, *P. argentea* and *P. judica* increased the aPTT at 50 mg/ml ((p =.05). Among of them, *P. argentea* achieved similar significant anticoagulation effect (p =.05) at all other studied concentrations (12.5 and 25 mg/ml). The recorded anticoagulation effects were concentration dependent in *U. urens*, *T. spicata* and *P. judica* (p =.05). Detailed results of aPTT of all examined plant species extracts are shown in Table 2 and Figure 2.

Table 2. The effect of the studied plant species extracts at different concentrations (50, 25 & 12.5 mg/ml) on aPTT values of five human platelets poor plasma samples.

Plant Species	Concentration (mg/ml)	aPTT (s)	*P value	**P value

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	50	71.59	.968	
Lupinus pilosus	25	32.28	1	.142
	12.5	27.56	1	
	50	206.23	.015	
Parietaria judaica	25	43.07	1	.004
	12.5	31.59	1	
	50	400	.001	
Paronychia argentea	25	388.52	.001	.174
	12.5	289.37	.001	
	50	36.6	1	
Ruta chalepensis	25	28.95	1	.398
	12.5	28.78	1	
	50	154.38	.362	
Satureja thymbra	25	126.68	.329	.377
	12.5	30.93	1	
	50	23.84	1	
Teucrium creticum	25	28.3	1	.24
	12.5	26.71	1	
	50	375.59	.001	
Thymbra spicata	25	177.02	.179	.002
	12.5	31.08	1	
	50	343.37	.001	
Urtica urens	25	193.05	.11	.017
	12.5	65.52	.861	
	50	140.05	.237	
Verbascum fruticulosum	25	29.42	1	.099
	12.5	27.84	1	

**p value = .05 was significant among the different studied plant species relative to the control (blood sample without plant extract). **p value = .05 was significant among the different concentrations of each examined plant species extract.*

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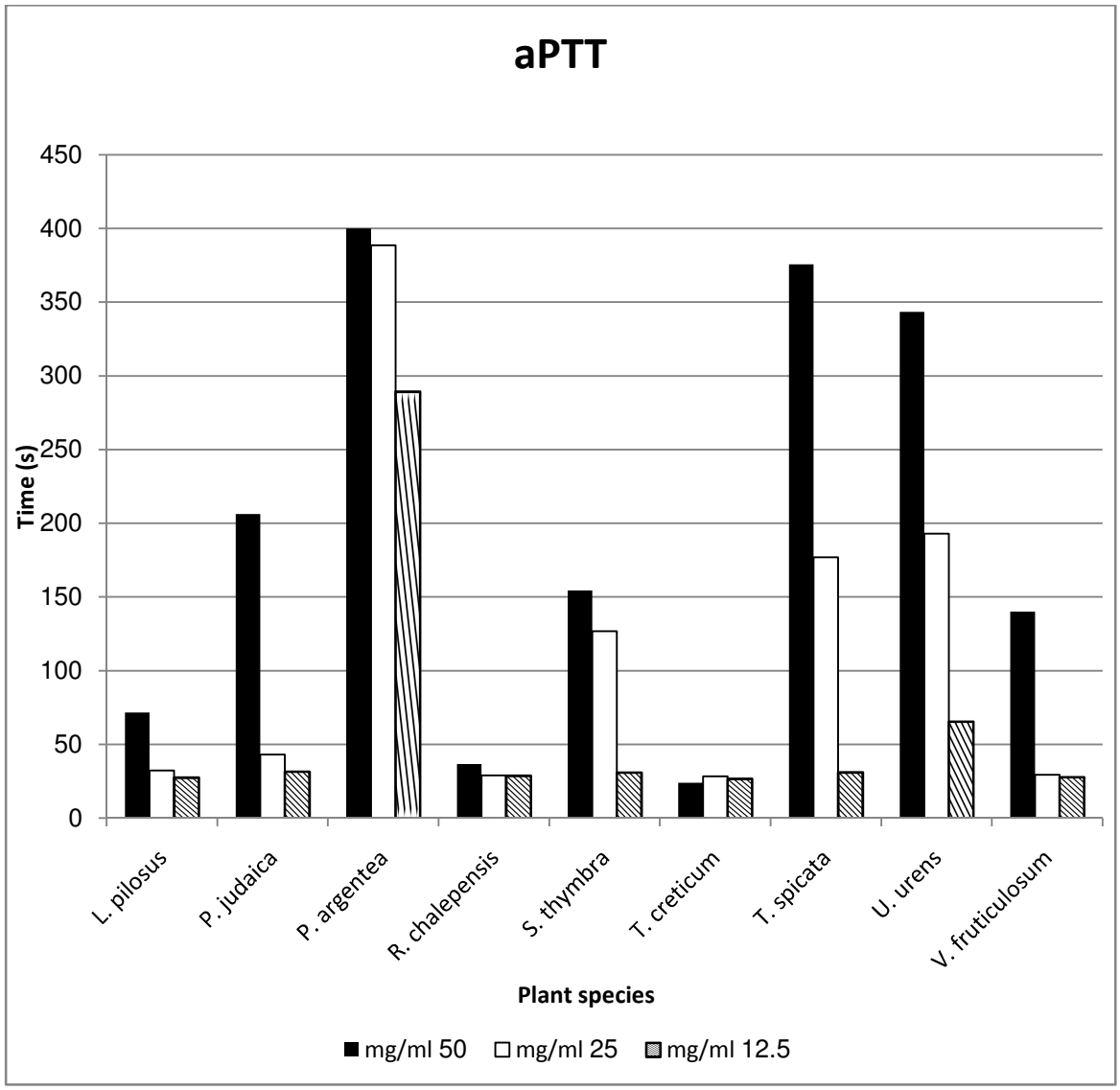

Fig. 2. The effect of the studied plant species extracts on aPTT values of five human platelets poor plasma samples at 50, 25 & 12.5 mg/ml concentrations.

4. DISCUSSION

The extrinsic (tissue factors) and/or the intrinsic (contact factors) pathway constitute the coagulation cascade revealing into blood haemostasis [26]. The standard clotting times for these two pathways are between 12.5 and 13.7 seconds for PT and between 31 and 39 seconds for aPTT [27]. Therefore, in comparison to the standard values of PT and aPTT, it is apparent that some of the examined plants in this study have had anticoagulation or coagulation effect on the examined blood samples.

Urtica urens extract prolonged PT at 50 mg/ml, while *T. spicata* one was at both 50 and 25 mg/ml. These out findings suggest that both plant extracts may have an inhibitory effect on the tissue clotting factors, which belong to the extrinsic pathway of the coagulation cascade [28, 29].

Absence of significant anticoagulation effect on the examined blood samples was observed by *S. thymbra* and *R. chalepensis* extracts. Nevertheless, they performed a concentration dependent effect at all examined concentrations on the PT, as the statistical analysis of the recorded data among the different

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examined concentrations was significant ($p = 0.05$). This suggests their possible anticoagulation effect at higher concentrations.

On the contrary for those plants, *P. argentea* demonstrated a marked decreasing effect on the PT at all its studied concentrations. It caused direct immediate clotting of the examined blood samples recording zero PT. As a result, a proposed presence of tissue factors like substances or activators to tissue thromboplastin could explain the thrombotic effect of *P. argentea* on the extrinsic pathway of the coagulation cascade [29, 30]. This out finding confirms the folkloric antihemorrhagic therapy use of some plants by herbalist [31].

Furthermore, *U. urens*, *T. spicata*, *P. argentea* and *P. judica* prolonged the aPTT relative to the control significantly ($p = 0.05$). This anticoagulation bioactivity was recorded at 50 mg/ml. The observed anticoagulation effect could be referred to the inhibition of the contact factors in the intrinsic pathway of the coagulation cascade [32], including the factors XII, XI, IX, and VIII [28, 29]. The obtained data in this study coincide with previous literatures in that some plant extracts have anticoagulation effect via increasing the aPTT [29, 33]. Among the previously mentioned effective plant species extracts on aPTT, only *P. argentea* did not show significant dose dependent manner ($p > 0.05$).

The greatest anticoagulation activity was seen in *U. urens* and *T. spicata* as they prolonged both PT and aPTT, indicating that both plant species could have inhibitory effect not only on the clotting factors in the intrinsic and extrinsic pathways, but also, those in the common pathways of the coagulation cascade. Therefore, the factors X, V, II and I could be susceptible for inhibition [32]. In contrast, the common pathway in the coagulation cascade is not vulnerable to *P. argentea* extract as it increased aPTT and decreased PT.

In overall the recorded results go along with literature in that biological substances in some plants (for example polysaccharide) can cause activation or inhibition to both the intrinsic and the extrinsic pathways [33]. In addition to that, many phytochemical substances attenuated the coagulation cascade and can inhibits or decrease the activity of tissue factors or thrombin [30].

As anticoagulant medicines can reduce the mortality and hospitalization, which result from the cardiovascular thrombotic diseases, screening of some medicinal wild plants in this study was conducted to provide scientific validation to their traditional medicinal use. Moreover, it could also lead to the discovery of new pharmacologically active drugs. The fundamental results of this research that some of the studied plant species extracts posses potent anticoagulation properties, which deduce the use of herbal preparations before undergoing any surgical procedure should be rather ceased. That can be referred to their possible anticoagulation effect, which may increase the risk of bleeding episodes. Moreover, in spite of the apparent anticoagulation effect of the examined plants *in vitro* as was proven in this study, it is not conclusive as *in vivo* studies will be required to determine their true physiological effect in combination to their cytotoxicity detection.

As the extraction of different ingredients from medicinal plant materials involves the use of various solvents based on their ability to extract bioactive compounds of different solubility and polarities [34], the use of other extract types of the non-effective plant species in this study may reveal different effects, which were not obtained here. As well as, when applied to the recorded effective ones may also show different bioactivity on the different blood parameters.

4. CONCLUSION

In conclusion, the effective examined plant species provide potential bioactivity from which anticoagulation or anti-bleeding drugs can be exploited. Therefore, further analysis is recommended for the identification and extraction of the effective ingredients in the effective recorded examined plant species. Moreover, the evaluation of the active plant extracts bioactivity mechanism on the coagulation cascade is suggested. In addition, it is recommended to identify the specific clotting factors in the coagulation cascade that are most sensitive to the action of these plants.

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CONSENT

As per international standard or university standard written patient consent has been collected and preserved by the authors.

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