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Investigation of the effect of selected edible and medicinal plants on *in-vitro* blood coagulation profile

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Abstract

Anticoagulation therapy represents a mainstay of treatment and prevention of cardiovascular diseases, which are the leading causes of mortality worldwide. In addition, several case reports of spontaneous bleeding occurred, linking the consumption of many dietary supplements during treatment with anticoagulants or in postoperative patients. This prompted our study, which was conducted on eleven well-known Egyptian medicinal plants, to highlight their effect on blood coagulation profile using Prothrombin time (PT) and activated partial thromboplastin time (aPTT) tests. Some of these plants showed exciting results that need a more in-depth evaluation of their anticoagulant activity as *Hibiscus sabdarifa* calyx, for its effect on PT. In addition, extracts of *Trifolium alexandrinum* aerial parts and *Pimpinella anismum* fruit were proved to affect aPTT. Therefore, postoperative or cardiovascular patients using herbal supplements should be cautioned about food-drug or herb-drug interactions and adjust their herbal medication regimen before surgery.

Keywords: Activated partial thromboplastin time**;** coagulation; Egyptian medicinal plants; *Hibiscus sabdarifa*; prothrombin time.

1. Introduction

Thromboembolic disorders, including pulmonary emboli and myocardial infarction, are the leading causes of mortality and morbidity (Moses, 2015). Many plants were recognized in folk medicine for their use in blood-related disorders; to prevent clot formation as anticoagulants or antiplatelets, but the mechanism of action and efficacy of most of them were not thoroughly studied (Cordier & Steenkamp, 2012). The well-known oral anticoagulant; warfarin was developed based on the molecular structure of dicoumarol in spoiled sweet clover (Moualla & Garcia, 2011; Stahmann *et al*., 1941).

Many plants have been reported to contain several potential antiplatelet and anticoagulant compounds. Some of these active plants are well-known edible ones, for example, onion and garlic (Liakopoulou-Kyriakides *et al*., 1985). Besides, clinically significant drug interactions were reported when certain herbal supplements were concomitantly used during warfarin therapy (Nutescu *et al*., 2006). As an example, ginkgo and ginseng present in many dietary supplements

showed several case reports of spontaneous bleeding during their use with warfarin because of their effects on platelets and coagulation (Vaes & Chyka, 2001).

The process of developing a novel anticoagulant drug is costly. Meanwhile, there is a pressing need for an oral cheaper yet more effective alternative. Currently, scientific evidence demonstrates that the intake of phytochemicals or dietary supplements with anticoagulant properties can minimize the risks of thromboembolic disorders. The *in-vitro* screening methods to evaluate newly developed anticoagulants obtained from natural and synthetic sources include the Prothrombin time (PT) and the activated partial thromboplastin time (aPTT). These two assays are currently employed within most laboratory testing for monitoring anticoagulant medication (Brace, 2001; Favaloro *et al*., 2011).

Two integrated procoagulant pathways are triggered upon vascular wall injury, namely, primary and secondary haemostasis. The direct pathway involves platelets activating a significant role in haemostasis and thrombosis (Gresele, 2013). The secondary path is represented by "blood coagulation", which is based on the properties of blood components; procoagulant proteins “coagulation factors”(Moore *et al*., 2016). This secondary hemostatic pathway comprises the (intrinsic) coagulation pathway initiated through vascular wall injury via the tissue factor (extrinsic) pathway (Favaloro *et al*., 2011). The two *in-vitro* assays used in our study represent these two pathways of secondary haemostasis.

The prothrombin time (PT) test estimates the extrinsic coagulation mechanism. It detects effects on coagulation factors II, V, VII and X. It is primarily used to monitor vitamin K antagonists (VKA) therapy that acts on three of these five factors and is the most commonly used anticoagulant medication. For this reason, this *in-vitro* test is the major haemostasis test performed in laboratories. Besides, most reagents in this assay contain a heparin neutralizing agent to increase specificity for VKA therapy and prevent the detection of heparin activity. The activated partial thromboplastin time (aPTT) *in vitro* test model acts on the components of the intrinsic and common pathways, including factors II, V, VIII, IX, FX, XI and XII (Osoniyi & Onajobi, 2003). This test is used mainly to monitor patients receiving unfractionated heparin (UH) and as a screening test for haemophilia A and B.

Egyptian flora has a wide molecular diversity of bioactive compounds. In the present study, eleven Egyptian medicinal plants previously reported affecting bleeding confirmed by *in-vitro* or *in-vivo* experiments were in vitro screened for their coagulation profile using (PT) and (aPTT) testing.

2. Materials and methods

2.1.Preparation of sample solutions:

Roots of *Glycyrrhiza glabra,* leaves of *Olea europaea*, aerial parts of *Trifolium alexandrinum* and *Medicago sativa* were collected from Alexandria, Egypt (table1). *Ammi visnaga, Ammi majus, Hibiscus sabdariffa, Linum usitatissimum, Matricaria recutita*, *Pimpinella anisum* and *Trigonella foenum-graecum* were purchased from a well-reputed herb store (Madi, Alexandria) in 2018 they are originated in Alexandria, Egypt. Still, the exact time of collection is not available. Plant samples

were kindly identified by Professor Dr Selim Zidan Heneidy, professor of Applied Ecology, Faculty of Science, Alexandria University. Voucher specimens; (GG106, OE107, TA108, MS109) have been deposited in the Pharmacognosy Department, Faculty of Pharmacy, Alexandria University. Each of the eleven plants was treated as follows; 15 g of dried powdered plant material was separately extracted with 70% ethanol three times, each with 150 ml using sonication at room temperature for 30 min. The combined filtrates were concentrated to dryness under vacuum. Dried plant extracts were dissolved in 10% DMSO in PBS, filtered using a 0.45 µ syringe filter and used in 2 concentrations (3 & 10 mg/ml).

Table 1. Site and date of collection of some plants

Plant	Site of collection	Date
Roots of *Glycyrrhiza glabra*	Smouha, Alexandria	from November to December 2018
leaves of *Olea europaea*	Borg-El-Arab, Alexandria	from October to November 2018
aerial parts of *Trifolium alexandrinum*	El-Awayed- Alexandria	from November 2018 to March 2019
Medicago sativa	Borg-el-Arab, Alexandria	December 2018

2.1. *In-vitro* prothrombin time (PT) and activated partial thromboplastin time (aPTT) testing

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured according to the procedure described in previous literature (Brown, 1988). All the experiments were carried out identically. EDTA at 10 mg/ml concentration was used as a positive control. The effect of the samples on bleeding was expressed as clotting times in seconds. Prothrombin time (PT) Platelet poor plasma was prepared by centrifuging citrated blood for 15 min at 4000 g. Samples were prepared by adding 0.5 ml of the prepared plant extract to 1 ml of platelet-poor plasma and then incubated for 5 min. at 37^{o}C (extract- plasma mixture). 200 µl of Thromboplastin calcium reagent (Hemostat thromboplastin-SI, Human, Diagnostica Stagio) after reconstitution and pre-warming was added to 100 µl of (extract- plasma mixture) while simultaneously starting a timer. Then gently shaken until the formation of a clot and clotting time are recorded. For the negative control, 10% DMSO in PBS was mixed with platelet-poor plasma and used instead of the sample extract.

2.1.1. Activated partial thromboplastin time (aPTT)

The (extract-plasma mixture) prepared as in the PT test was mixed with the prewarmed aPTT reagent (aPPT-EL, Human, Diagnostica Stagio) in an equal amount (100 µl). Then, incubation at 37^{o}C for 3 min. I was followed by the addition of 100 µl of the prewarmed calcium chloride solution (0.02 M) while, at the same time, starting a timer. Next, the tube was gently shaken while still in the water bath every 5 s. after 20 s, it was removed and gently shaken until a clot was observed and the time recorded. The negative control used is 10% DMSO in PBS.

2.2. Statistical analysis

The statistical analysis was performed using Graphpad Prism® (V. 6.01) software. All the experiments were carried out identically, and the measured data are expressed as the means ±

standard deviation. In addition, an unpaired student t-test was used to determine the statistical significance between the control and treatment groups.

2.3. Ethical review

The study received ethical approval from the Ethics Committee of the faculty of pharmacy because of the very small volume of blood used; only oral consent of blood donors was obtained after explaining the aim of the study.

3. Results

The standard clotting time is 12.5 to13.7 s for the PT test and between 31 and 39 s for aPTT. Values deviated from these standard times indicate an effect on the coagulation either as an anticoagulant; with prolonged clotting times, or as a coagulant, with rapid clot formation (Cordier *et al*., 2012). The eleven plants proved their anticoagulant effects as concluded from the differences in their clotting times compared to the control in the PT and aPTT *in-vitro* testing (Table 2). It was observed that almost all the tested plants showed a significant effect in aPTT testing rather than PT test at the two concentrations used: (3& 10 mg/ml)

Table 2. Effect of ethanolic plant extracts on PT and aPTT clotting times

Sample	Concentration (mg/ml)	PT (Seconds)#	aPTT (Seconds)#
Normal plasma	-	12.80	26.58
NegativeControl (10%DMSO)	-	14.65± 0.07	56.1±1.06
Positive control (EDTA)	10	23.25± 0.35	506.4±0.74
Ammi majus[b]	3	14.60±0.14	31.5±0.42**
(AM)	10	19.2±0.14**	35.2±1.41**
Ammi visnaga	3	18.25±0.78	49.1±0.14*
(AV)	10	18.75±0.21*	57.6±0.42
Glycyrrhiza glabra[b]	3	16.6±0.28	35.3±1.48**
(GG)	10	16.65±0.21*	30.1±0.85**
Hibiscus sabdariffaa[b]	3	20.35±0.07***	41.10±0.14**
(HS)	10	no clotting*	44.8±0.99**
Linum usitatissimum[b]	3	18.95±0.64	80.35±0.78**
(LU)	10	15.90±0.14*	111.6±0.85***
Matricaria recutita[b]	3	20.65±0.07***	75.6±0.07**
(MC)	10	17.35±0.64	67.8±0.57**
Medicago sativa[a]	3	11.20±0.14**	15.1±1.2***
(MS)	10	21.55±0.07***	55.2±2.55
Olea Europae[a]	3	16.00±0*	32.4±0.42***
(OE)	10	16.65±0.21*	53.7±1.84
Pimpinella anisum[b]	3	13.80±0.28	75.85±1.48**
(PA)	10	13.45±0.07**	91.25 ±2.19**
Trifolium alexandrinum[b]	3	15.55±0.35	76.40±1.41**
(TA)	10	15.65±0.64	116.6±1.84***
Trigonella foenum-graecum[b]	3	14.10±0.42	36.1±0.42**
(TF)	10	19.50±0.42*	89.4±2.26**

#Values are expressed as the means of 2 replicates ± SD, values statistically significant when compared to negative control using unpaired t-test: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$

[a] plants with significant effects on PT. [b] plants with substantial impacts on aPTT

3.1. Prothrombin time (PT) testing

In PT testing, only three plants (*Olea europaea*, *Hibiscus sabdariffa* and *Medicago sativa*) showed significant effects on the clotting times compared to control when tested at two concentrations suggesting dose-dependent activity (Figure 1). Additionally, seven plants gave substantial observed impacts for one of the two concentrations (Table 2). These effects mainly prolonged clotting times except for *Pimpinella anisum*, which significantly showed shorter clotting times compared to control (13.45 s) at concentration10 mg/ml.

The highest effect was manifested by *H. sabdariffa* extract as it showed prolongation of the clotting time at a concentration of 10 mg/ml where the blood did not clot.

M. sativa extract also increased the PT at the higher concentration tested as it gave a bleeding time of 21.55 s, while at a lower concentration, bleeding time (11.20 s) wasn't affected but was lower than the time measured for normal plasma (12.80 s).

Change in clotting times for *O. europaea* extract showed minimal difference in increasing the concentration used.

T. foenum-graecum, A. majus, A. visnaga, G. glabra and L. usitatissimum showed significant prolongation of clotting times (19.50 s, 19.20 s, 18.75 s, 16.65 s and 15.90 s, respectively) at the highest concentration used). On the contrary, *M. Recutita* is the sole extract that gave longer times when tested at 3mg/ml *(*20.65 s).

3.2. Activated partial thromboplastin time (aPTT) testing

All the tested plant extracts showed statistically significant differences in their clotting times compared to the control in aPTT test (Figure 2, Table 2). In addition, eight of the examined plant extracts had effects in the two concentrations used, while the remaining three plants observed significant effects for one concentration only.

T. alexandrinum, L. usitatissimum, P. anisum, and T. foenum-graecum extracts showed the highest effects in prolongation of the clotting times (116.60 s, 111.60 s, 91.25 s and 89.40 s, respectively) at the highest concentration tested. *M. Recutita* extract at 3 mg/ml also prolonged aPTT significantly (75.6 s), while higher concentration at 10 mg/ml showed shorter times. On the other hand**,** *G. glabra, H. sabdariffa* and *A. majus* extracts showed shorter clotting times than the control used (56.10 s). Besides, shorter clotting times were observed for the three plants: *Olea europaea*, *Ammi visnaga* and *Medicago sativa.* These clotting times were significant at the lower concentration, 3 mg/ml being 32.40 s, 49.10 s and 15.10 s, respectively.

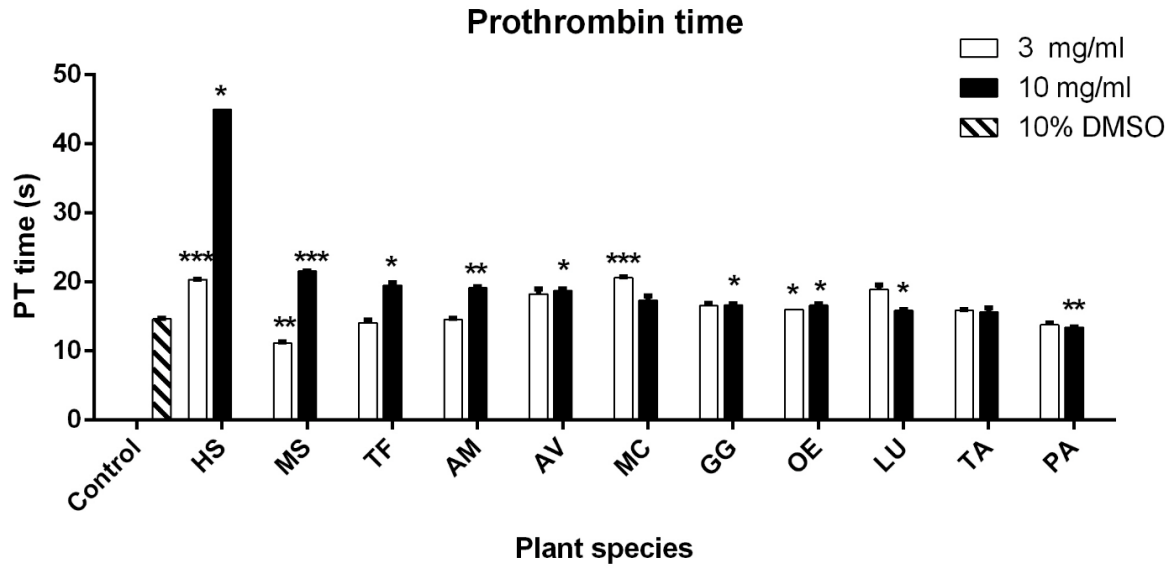

Fig. 1. Effect of plants ethanolic extracts on PT *in-vitro*. *p< 0.05, **p < 0.01 and ***p< 0.001 compared to negative control (10%DMSO).
Bars without symbols refer to non-significant results

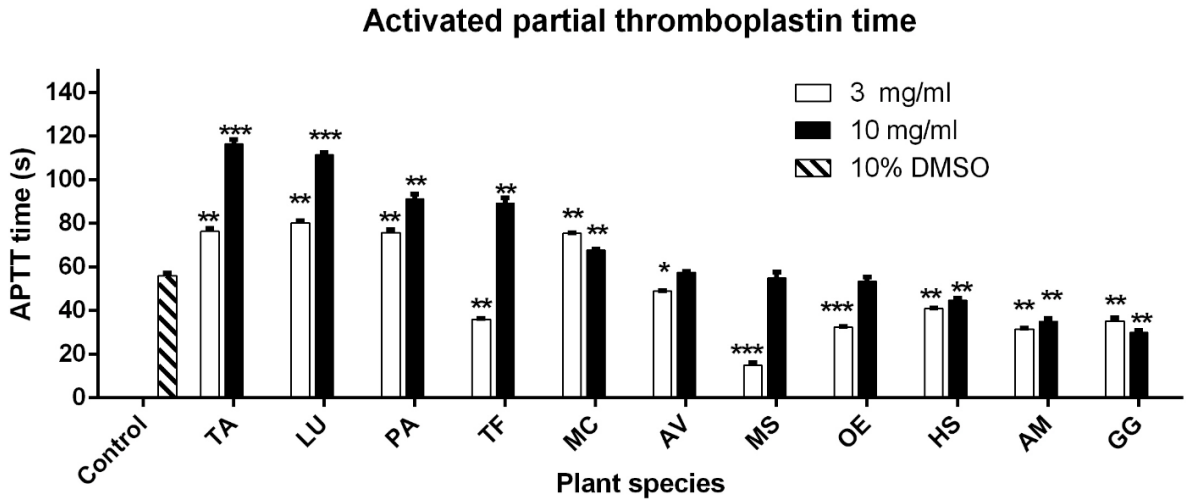

Fig. 2. Effect of plants ethanolic extracts on aPTT *in-vitro*. *p<0.05, **p< 0.01 and ***p< 0.001 compared to negative control (10% DMSO).
Bars without symbols refer to the non-significant result

4. Discussion

The results indicated that some of the tested plant extracts affected different coagulation factors through their effects on PT and aPTT compared to the control. Our findings agreed with some plants' previously reported anticoagulant activities, as in the case of *O. Europea,* where a previous study showed that oral supplementation of the plant leaves extract possessed a significant prolongation of PT with no effect on aPTT (Dub & Dugani, 2013).

Anticoagulant effects of *G. glabra* are well known, and their impact on PT and aPTT has been reported in many studies (Braun and Cohen, 2015; Nakagawa *et al*., 2008). Some used it as a positive control with other plant extracts (Aalikhani Pour *et al*., 2016). In our study, clotting time found for *G. glabra* was very close to the previously reported times for PT test *in-vitro (*17.30 s) (Aalikhani Pour *et al*., 2016). However, the aPTT time was shorter than the values reported in the literature (82.66 s at a concentration of 0.1 mg/ml). Hence, further examination of the effect of *G. glabra* extract on aPTT using a wider concentration range is highly recommended.

T. alexandrinum extract showed strong activity towards aPTT even at concentrations as low as 3 mg/ml, with no effect on PT. The obtained aPTT activity, as opposed to the previously obtained results, suggested that the plant did not affect the coagulation times (TT, PT or aPTT) (Kolodziejczyk-Czepas *et al*., 2018). Differences in the sample preparation could be one of the reasons for the differences in the results. In our study, we used the total ethanolic extract in *vitro* testing; compared to phenolic fractions in the mentioned study.

The prolongation of PT observed for *H. sabdariffa* dried calyx extract is an interesting finding that needs a further examination of the exact mechanism and phytoconstituents responsible for this activity. Previous studies reported *in-vivo* antiplatelet effects for dried Hibiscus calyx extract (Ali *et al*., 2016).

Previous studies showed that *M. Recutita* extract; (especially the aqueous one) exhibited significant *in-vitro* antiplatelet aggregation (Memariani *et al*., 2018). This antiplatelet activity was induced by polysaccharide–polyphenolic conjugates present in the dry flowering parts of the plant (Bijak *et al*., 2013). luteolin, a flavone presents in chamomile, inhibited thrombin (factor II) and FXa activity. It also prolonged aPTT and PT, reaching 81.0 s and 16.2 s, respectively, when tested at a concentration of 10 µg. This could explain the anticoagulant activity observed in our study for *M. Recutita* extract, as the aPTT was more affected by the plant extract than PT (Choi *et al*., 2015). Our findings revealed that *M. Recutita* extract is an effective anticoagulant using these two *in-vitro* tests, especially at low concentrations (3 mg/ml).

The majority of the results on flaxseed showed that its ingestion or its oil does not influence platelet aggregation (Rodriguez-Leyva *et al*., 2010). Only a few studies have shown that the oil inhibited platelet aggregation induced by thrombin and fibrinogen (Prasad, 2009). Recent *in-vitro* studies indicated that a phenolic extract of the seed has moderate activity on both coagulation pathways, as indicated by PT and aPTT testing (Boukeria *et al*., 2020). Moreover, flax seed proteins were evaluated for their effects on coagulation disorders. The results showed that this protein preparation prolonged the clot formation process of only aPTT but not PT at 0.1 mg (Nandish *et al*., 2018). Our findings confirmed these results regarding the aPTT activity.

A literature survey regarding *T. foenum-graecum* showed that the plant extract inhibited platelet aggregation in *an in-vivo* rat model (Ulbricht *et al*., 2008). Moreover, the 5% aqueous fenugreek extract inhibited the coagulation process *in-vitro* and prolonged PT in a dose-dependent manner, reaching 23.60 s when 75 µl was used (Taj Eldin *et al*., 2013). Our findings came from these studies as the plant extract showed significant prolonged PT (19.50 s) at 10 mg/ml. Moreover, significantly longer clotting times were observed in our study in aPTT test. Thus, our results partly explain the increased bleeding risk reported for concomitant use of fenugreek preparations with warfarin (Lambert & Cormier, 2001).

The results obtained for *M. sativa* came from earlier studies where the plant aqueous and methanolic extracts had aPTT clotting times of 50.40 s and 51.20 s, respectively, in previous studies (Cordier *et al*., 2012).

A. visnaga, A. majus and P. anisum are three plants belonging to the family Apiaceae famous for their content of coumarins, well-known anticoagulants (Arora & Mathur, 1963). Previous studies showed that *A. visnaga* improves blood supply to coronary smooth muscles (Khalil *et al*., 2020).To our knowledge, anticoagulant and antiplatelet activity of *A.visnaga* and *A. majus* haven't been previously evaluated. Our findings revealed the significant anticoagulant activity of *P. anismum* demonstrated by the prolonged aPTT. In contrast, *A. majus* extract showed shorter clotting times observed in aPTT, indicating coagulating activity. The same extract showed anticoagulant properties in PT manifested by longer clotting time (19.20 s) at a concentration of 10 mg/ml. *A. visnaga* is the only plant that didn't show significant effects in both *in-vitro* anticoagulant models.

5. Conclusion

Our *in-vitro* anticoagulant study gave further evidence on the activity of some medicinal plants known for blood clot management. Some of these plants showed exciting results that need a more in-depth evaluation of their anticoagulant activity as *H. sabdarifa* calyx extract, for its effect on PT. In addition, extracts of *T. alexandrinum* aerial parts and *P. anismum* fruit were proved to affect aPTT.

It should be noted that plants containing coumarins that didn't show anticoagulant effects in our study will need further investigations using *in-vivo* models for coagulation since they exert their anticoagulant properties by inhibiting the vitamin K epoxide reductase complex, which can be monitored *in-vivo* (Hildebrandt and Suttie, 1982; Kasperkiewicz *et al*., 2020).

In general, plant extracts suppressing the extent of coagulation in PT are suggested to have an effect on one or more of the coagulation factors involved in the extrinsic pathway. Plants prolonging the aPTT are considered to act on coagulation factors VIII, IX, XI and XII of the endogenous coagulation (Giddens, 2015).

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