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 is 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 interesting results that need 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. Post operative or cardiovascular patients using herbal supplements should be cautioned about food-drug or herb-drug interactions and to adjust their herbal medication regimen before surgery.

Keywords: Activated partial thromboplastin time**;** Coagulation; *Hibiscus sabdarifa*; Prothrombin time; Egyptian medicinal plants.

1. Introduction:

Thromboembolic disorders including pulmonary emboli, and myocardial infarction are the leading causes of mortality and morbidity (Moses and Deisy 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 fully studied (Cordier and Steenkamp 2012). In fact, the well-known oral anticoagulant; warfarin was developed based on the molecular structure of dicoumarol present in spoiled sweet clover (Moualla and Garcia 2011).

Many plants have been reported to contain several potential anti-platelet and anti-coagulant 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 therapy because of their effects on platelets and coagulation (Vaes and Chyka 2001)

The process of developing a novel anticoagulant drug is costly meanwhile there is a pressing need for an oral cheaper yet effective alternative one. At present, there are scientific evidences demonstrating that the intake of phytochemicals or dietary supplements with anti-coagulant properties can minimize the risks of thromboembolic disorders. The *in-vitro* screening methods to evaluate newly developed anticoagulants obtained from natural sources as well as synthetic 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 (Favaloro et al. 2011).

Upon vascular wall injury, two integrated pro-coagulant pathways are triggered namely, primary, and secondary haemostasis. The primary pathway involves activation of platelets that possess major role in haemostasis and thrombosis (Gresele 2013). The secondary pathway is represented by "blood coagulation" which is basically based on 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.

Prothrombin time (PT) test gives an estimate for the extrinsic coagulation mechanism. It detects effects on coagulations factors II, V, VII and X. It is primarily used to monitor vitamin K antagonists (VKA) therapy that act on three out of these five factors and is the most common used anticoagulant medication. For this reason, this *in-vitro* test is the major haemostasis test performed in laboratories. Besides, most reagents utilized in this assay contain a heparin neutralizing agent to increase specificity for VKA therapy and prevent detection of heparin activity. 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 and Onajobi 2003) .This test is used mainly to monitor patients receiving unfractionated heparin (UH). and as a screening test for haemophilia A and haemophilia B.

Egyptian flora has a wide molecular diversity of bioactive compounds. In the present study, eleven Egyptian medicinal plants previously reported to affect bleeding confirmed both 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 well reputed herb store (Madi, Alexandria) in 2018 they are originated in Alexandria, Egypt, but 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 were 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 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

2.2. *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 in a duplicate

manner. EDTA at concentration 10 mg/ml was used as 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 centrifugation of 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°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 recorded. For the negative control, 10% DMSO in PBS was mixed with platelet poor plasma and used instead of the sample extract.

2.2.1. Activated partial thromboplastin time (aPTT):

The (extract- plasma mixture) prepared as in PT test was mixed with the pre-warmed aPTT reagent (aPPT-EL, Human, Diagnostica Stagio) in an equal amount (100 µl). Then, incubation at 37°C for 3 min. following by the addition of 100 µl of the prewarmed calcium chloride solution (0.02 M) while on the same time, starting a timer. 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.

2.3.Statistical analysis:

The statistical analysis was performed using Graphpad Prism® (V. 6.01) software. All the experiments were carried out in a duplicate manner and the measured data is expressed as the means ± standard deviation. Unpaired student t-Test was used to determine the statistical significance between control and treatment groups.

2.4.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 normal clotting time is from 12.5 to13.7 s for PT test, and between 31 and 39 s for aPTT. Values deviated from these standard times indicate an effect on the coagulation either as anticoagulant; with prolonged clotting times or as 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 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
Negative Control (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 europaea[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 significance 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 significant effects 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 as compared to control when tested at two concentrations suggesting dose dependant activity (**Fig 1**). Additionally, seven plants gave significant effects that were observed for one of the two concentrations (**Table 2**). These effects were mainly prolongation of clotting times except for *Pimpinella anisum* that 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 concentration of 10 mg/ml where the blood did not clot at all.

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

Change in clotting times for *O. europaea* extract showed minimal change on 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 (**Fig 2**, **Table 2**). Eight of the examined plant extracts had effects in the two concentrations used while the remaining three plants gave significant effects observed 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 tested 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*. NC: no clotting, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to negative control (10%DMSO). Bars without symbols refer to non-significant results

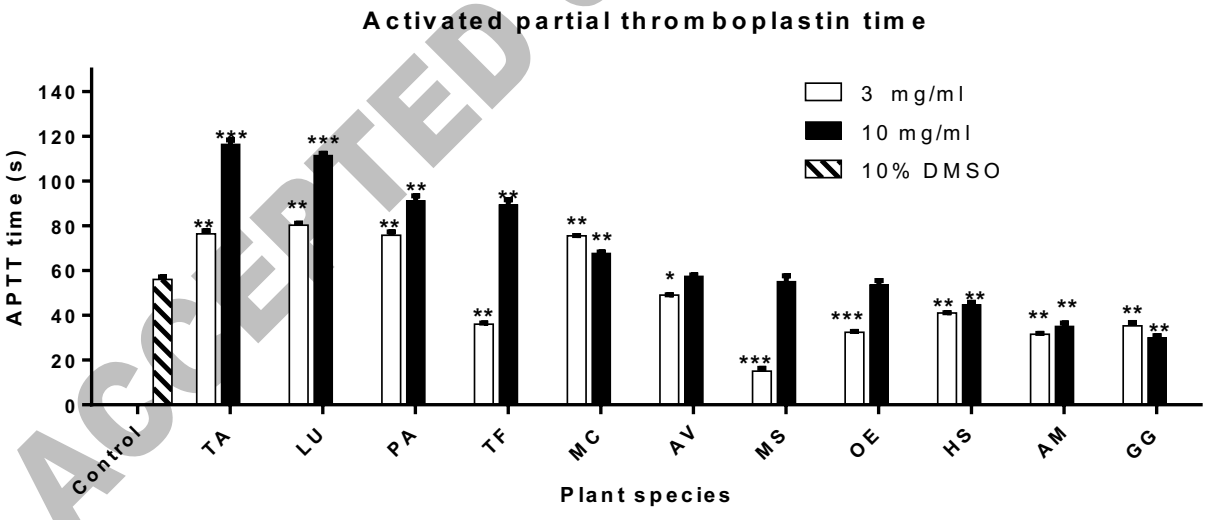

Fig 2: Effect of plants ethanolic extracts on aPTT *in-vitro*. NC: no clotting, *$p<0.05$, **$p< 0.01$ and ***$p< 0.001$ compared to negative control (10% DMSO). Bars without symbols refer to non-significant results

4. DISCUSSION

The obtained results indicated that some of the tested plant extracts affecting different coagulation factors through their effects on PT and aPTT compared to the control. Our findings came in agreement with the previously reported anticoagulant activities of some plants 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 and Dugani 2013).

Anticoagulant effects of *G. glabra* are well known and its effects on PT and aPTT were reported in many studies (Braun and Cohen 2015; Nakagawa et al. 2008). Some of which used it as a positive control with other plants 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 literature (82.66 s at concentration 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 while no effect on PT was observed. The obtained aPTT activity was opposing to the previously obtained results that suggested that the plant did not affect the coagulation times (TT, PT or aPTT) (Kolodziejczyk-Czepas et al. 2018). Difference in the sample preparation could be one of the reasons for the differences in the results as in our study we used the total ethanolic extract in *in vitro* testing; compared to using phenolic fractions in the mentioned study.

The prolongation of PT observed for *H. sabdariffa* dried calyx extract is an interesting finding that needs 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* anti platelet aggregation (Memariani et al. 2018). This anti platelet activity was induced by polysaccharide–polyphenolic conjugates present in the dry flowering parts of the plant (Bijak et al. 2013). luteolin, a flavone present 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 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 effective anticoagulant using these two *in-vitro* tests especially at low concentration (3 mg/ml).

The majority of the results on flaxseed showed that its ingestion or its oil do not influence platelet aggregation (Rodriguez-Leyva et al. 2010). Only few studies have shown that the oil inhibited platelet aggregation induced by both 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 2020). Moreover, flax seed proteins were evaluated for their effects on coagulation disorders and 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

Literature survey regarding *T. foenum-graecum* showed that the plant extract inhibited platelet aggregation in *in-vivo* rat model (Ulbricht et al. 2008). Moreover, the 5% aqueous extract of fenugreek was found to inhibit 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 in accordance with these studies as the plant extract showed significant prolongation of PT (19.50 s) at 10 mg/ml. Moreover, significantly longer clotting times were observed in our study in aPTT

test. Thus, our results explain in part the increased bleeding risk reported for concomitant use of fenugreek preparations with warfarin(Lambert and Cormier 2001).

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

A. visnaga, A. majus and P. anisum are three plants belonging to family Apiaceae famous for its content of coumarins, well known anticoagulants (Arora and Mathur 1963). Previous studies showed that *A. visnaga* improves blood supply to coronary smooth muscles (Khalil et al. 2020).To the best of 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 concentration 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 a further evidence on the activity of some medicinal plants known for blood clot management. Some of these plants showed interesting results that need 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).

In general, plants extracts suppressing the extent of coagulation in PT are suggested to possess 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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Conflict of interest disclosure:

The authors declare no conflict of interest.

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