Brazilian Journal of Biology
ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

https://doi.org/10.1590/1519-6984.219186
Original Article

In vitro haemostatic efficacy of aqueous, methanol and ethanol plant extracts of three medicinal plant species in Palestine

G. Omar[a]*, *L. Abdallah*[b], *A. Barakat*[b], *R. Othman*[c] and *H. Bourinee*[c]

[a]Department of Biology and Biotechnology, Faculty of Science, An-Najah National University, Nablus, Palestine

[b]Department of Statistics, Faculty of Science, An-Najah National University, Nablus, Palestine

[c]Department of Medical Laboratory Sciences, Faculty of Medicine and Health, An-Najah National University, Nablus, Palestine

*e-mail: ghaderomar@najah.edu

Received: January 29, 2019 – Accepted: June 26, 2019 – Distributed: November 30, 2020
(With 2 figures)

Abstract

The haemostatic efficacy of different extract types of *Satureja thymbra* L., *Thymbra spicata* L. (Lamiaceae) and *Verbascum fruticulosum* Post. (Scrophulariaceae) was evaluated in this study via the Prothrombin time (PT) and Activated partial thromboplastin time (aPTT) analysis. Aqueous, methanol and ethanol extracts of the examined plant species leaves were prepared to a final concentration 50 mg/mL. *In vitro* PT and aPTT assays were conducted on normal platelet poor plasma blood samples by a digital coagulation analyzer. The obtained results revealed anticoagulation activity of all investigated plant species with observed variations among them. The aqueous and ethanol extracts of *T. spicata* as well as the aqueous extract of *S. thymbra* prolonged PT values significantly ($p < 0.05$). While, all *V. fruticulosum* extract types have had no significant effect on the PT values. The recorded aPTT data showed that all aqueous extracts have had a significant effect on the blood haemostasis as they increased aPTT values in all plant species under study. Out of which, both the ethanol and methanol extracts of *T. spicata* and methanol extract of *S. thymbra* showed similar effect. Of great concern, it was clearly noticed that the aqueous and ethanol extract of *T. spicata* and the aqueous extract of *S. thymbra* possess the strongest anticoagulation effect as they increased both PT and aPTT values significantly relative to the control ($p < 0.05$). The variable anticoagulation bioactivity among the studied plant species could be referred to the various solvents degrees of solubility of different phyto-constituents. Thus, the efficacy of the plant species extracts evaluation as anticoagulants or coagulants were related to the plant species and to the solvent of extraction.

Keywords: medicinal plants, haemostasis, plant extract, Palestine.

Eficácia hemostática *in vitro* de extratos aquosos, metanólicos e etanólicos de três espécies de plantas medicinais na Palestina

Resumo

A eficácia hemostática de diferentes tipos de extrato de *Satureja thymbra* L., *Thymbra spicata* L. (Lamiaceae) e *Verbascum fruticulosum* Post. (Scrophulariaceae) foi avaliada neste estudo pelo tempo de protrombina (TP) e tempo de tromboplastina parcial ativada (TTPa). Os extratos aquosos, metanólicos e etanólicos das folhas das espécies de plantas examinadas foram preparados para uma concentração final de 50 mg/mL. Os ensaios de TP e TTPa *in vitro* foram realizados em amostras normais de sangue, pobre em plaquetas, por um analisador de coagulação digital. Os resultados obtidos revelaram atividade anticoagulante de todas as espécies de plantas investigadas, com variações observadas dentre elas. Os extratos aquosos e etanólicos de *T. spicata* e o extrato aquoso de *S. thymbra* prolongaram significativamente os valores de TP ($p <0,05$). Entretanto, todos os tipos de extratos de *V. fruticulosum* não tiveram efeito significativo sobre os valores de TP. Os dados registrados do TTPa mostraram que todos os extratos aquosos tiveram um efeito significativo na hemostase do sangue, pois aumentaram os valores de TTPa em todas as espécies de plantas em estudo. Dos quais, ambos os extratos etanólicos e metanólicos de *T. spicata* e o extrato metanólico de *S. thymbra* mostraram efeito semelhante. De grande preocupação, notou-se claramente que os extratos aquoso e etanólico de *T. spicata* e o extrato aquoso de *S. thymbra* apresentam efeito anticoagulante mais forte, aumentando os valores de TP e TTPa significativamente em relação ao controle ($p <0,05$). A variável bioatividade anticoagulante dentre as espécies vegetais estudadas pôde ser referida aos vários graus de solventes de solubilidade de diferentes fitoconstituintes. Assim, a eficácia da avaliação de extratos de espécies vegetais como anticoagulantes ou coagulantes foi relacionada às espécies vegetais e ao solvente de extração.

Palavras-chave: plantas medicinais, hemostase, extrato vegetal, Palestina.

1. Introduction

Cardiovascular diseases are the leading cause of death in the world, that arise from the conflicts associated with blood coagulation (Rang et al., 2007; Buch et al., 2010). Coagulation cascade occurs either via the tissue factors (extrinsic pathway) or the contact factors (intrinsic pathway) (Palta et al., 2014). Increased coagulation is associated with several cardiovascular diseases including coronary heart disease and hypertension (Mekhfi et al., 2004). While decreased coagulation leads to prolonged bleeding time which results either from certain diseases like haemophilia or from drugs like aspirin (Vane and Botting, 2003).

Medicinal plants were and still used traditionally as protective natural medication (Sargin et al., 2015). Epidemiological and ethnomedical studies revealed that the risk of several diseases in general and cardiovascular ones in particular are reduced by a high consumption of medicinal plants (He et al., 2007; Sargin et al., 2015). The role of the plants bioactive compounds that provide this protection is a hot spot of research (He et al., 2007). For example, the flavonoids were proved that they demonstrate beneficial effects on cardiovascular risk factors including blood pressure, endothelial function, platelets function and cholesterolemia (Hooper et al., 2008). Also the salicylic acid which is another extracted phytochemical from *Salix alba* L., that is transformed into antiplatelet drug known by aspirin (Vane and Botting, 2003).

Since the concern towards the ethno-medicinal scientific justification, as well as, the over whiling seeking for remedies devoid for unfavorable side effects have prompted the fascination growth for natural haemostatic agent's discovery. Accordingly, novel coagulants or anticoagulants can be found in other plant species. On the other hand, folkloric haemostasis therapy use of some medicinal plants by herbalists is applied in several countries, one of which is Palestine. Therefore, previous studies were conducted to evaluate this issue. In 2014, *Viscum album* extracts from olive and almond host plants were proved to have significant prolongation effect on PT and aPTT (Abualhasan et al., 2014). Later on, another *in vitro* study showed that the ethanol extracts of *Urtica urens*, *Parietaria judica*, *Satureja thymbra*, *Thymbra spicata*, *Teucrium creticum*, *Verbascum fruticulosum*, *Lupinus pilosus*, *Paronychia argentea*, and *Ruta chalepensis* had either anticoagulation or coagulation effect on the examined blood samples (Omar et al., 2017). A wide spectrum of different phytochemicals could be extracted using various solvents due to variations in their solubility and polarity (Liu, 2003). As a result, the use of other extract types of the non-effective plant species, as well as the recorded effective ones may reveal different bioactivity on the different blood parameters. From this point of view, this research popped out to examine the haemostatic efficacy of different extract types of *Satureja thymbra* L., *Thymbra spicata* L. (Lamiaceae) and *Verbascum fruticulosum* Post. (Scrophulariaceae).

2. Material and Methods

2.1. Plant materials

The three plant species (*Thymbra spicata* L., *Satureja thymbra* L. and *Verbascum fruticulosum* Post) were collected and identified by Ghadeer Omar, Department of Biology & Biotechnology, Faculty of Science, An-Najah National University; Palestine. Representative samples of the collected plant species under study were pressed till drying, treated chemically, mounted on herbarium sheets, provided with voucher numbers, and then they were deposited at An-Najah National University herbarium. The aerial parts of the 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 sterile boiled distilled water 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 freeze-drying. The obtained powder of each of the plant species was dissolved in distilled water to a final concentration equal to 50 mg/mL. However, for alcoholic extraction, ten grams of each plant powder were soaked in 100 mL of (70%) ethanol or methanol for one week with interval shaking. Then the same steps of the aqueous extraction procedure were repeated, except that (1%) dimethyl sulfoxide (DMSO) was used as a solvent and evaporation was performed by a rotary evaporator (Omar et al., 2017).

2.3. Blood sample preparation

Ten healthy volunteers were not under any medication and not smokers were asked to give blood samples. 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) (Saluja et al., 2011). 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 duplicates. The negative controls for PT and aPTT assays were distilled water for aqueous extract and 1% DMSO for alcoholic extracts.

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 for 5 min at 37 °C. 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 for 2 min at 37 °C. Then 50 µL aPTT reagent (Human, Germany) was added and incubated for further 3 min at 37 °C. 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 extracts relative to the control. P value < 0.05 was considered to be significant.

3. Results and Discussion

The examined blood samples were considered as representative samples for this study as no observed individual variations ($p < 0.01$) in PT and aPTT data. Based on the obtained results, most of the studied plant extracts showed an effect on coagulation cascade by increasing PT or aPTT time or both (Table 1). The aqueous and ethanol extracts of *T. spicata* as well as the aqueous extract of *S. thymbra* prolonged PT values significantly ($p < 0.05$). While, their other extract types and all *V. fruticulosum* extract types have had no significant effect on the PT values (Figure 1). The recorded aPTT data showed that all aqueous extracts had a significant effect on the blood haemostasis as they increased aPTT values in all plant species under study. Similarly, both the ethanol and methanol extracts of *T. spicata* and methanol extract of *S. thymbra* showed same effect (Figure 2). Of great concern, it was clearly noticed that the aqueous and ethanol extract of *T. spicata* and the aqueous extract of *S. thymbra* had the strongest anticoagulation effect as they increased both PT and aPTT values significantly relative to the control ($p < 0.05$).

The purpose of this study was to evaluate the haemostatic efficacy of different extract types of *T. spicata*, *S. thymbra* and *V. fruticulosum* leaves. The haemostatic effect was determined by PT and aPTT assays. The prothrombin time (PT) and activated partial thromboplastin time (aPTT) are indicators for coagulation since they are influenced by the susceptibility of the tissue factors (extrinsic) and contact factors (intrinsic) pathways, respectively (Palta et al., 2014). Standard clotting times for these two pathways are between 12.5 and 13.7 s for PT and between 31 and 39 s for aPTT (Lentner, 1984).

As the aqueous and ethanol extracts of *T. spicata* and the aqueous extract of *S. thymbra* prolonged both PT and aPTT values, they could be considered to have the strongest anticoagulation effect among other studied ones. This bioactivity could be referred to their act not only on the extrinsic (tissue factors) or intrinsic (contact factors) pathways, but also on the common one. On the other hand, the intrinsic (contact factors) pathway of the coagulation cascade was inhibited by the methanol extracts of *T. spicata*, and *S. thymbra* as well as the aqueous extract of *V. fruticulosum* by increasing only aPTT.

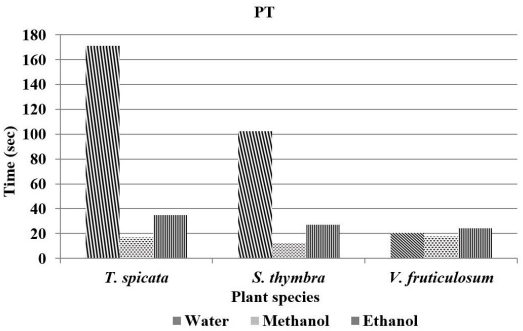

Figure 1. Prothrombin time (PT) values of the different examined plant species extracts types.

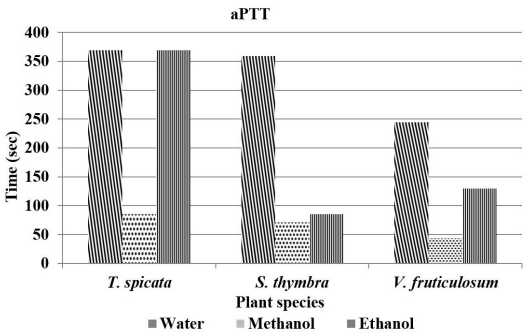

Figure 2. Activated partial thromboplastin time (aPTT) values of the different examined plant species extracts types.

Table 1. Prothrombin time (PT) and Activated partial thromboplastin time (aPTT) values of the studied plant species different extract types at 50 mg/mL, illustrating the statistical P values.

Plant Species	Extract	PT (sec)	P value	aPTT (sec)	P value
T. spicata	Water	171.2	0.001	369.4	0.001
	Methanol	16.8	0.891	49.4	0.001
	Ethanol	35	0.002	369.5	0.001
S. thymbra	Water	102.4	0.001	359.2	0.001
	Methanol	12.4	0.999	70.8	0.001
	Ethanol	26.9	0.065	84.9	0.362
V. fruticulosum	Water	20.8	0.204	244.2	0.001
	Methanol	17.7	0.412	85.2	0.446
	Ethanol	24.3	0.082	129.5	0.185

Based on the obtained results, the decrease in the coagulation activity is mediated through the inhibition or decrease of the activity of several factors including tissue factors, thrombin, vitamin K-epoxide reductase and other clotting factors (Preusch and Smalley, 1990; Lee et al., 2004; Zhang et al., 2008). Therefore, the evaluation of those active plant extracts bioactivity mechanism on the coagulation cascade is required.

The essential oils extracted from *T. spicata* and *S. thymbra* leaves (and flowering tops) were estimated during the growing season leading to that the concentration of the phenolic constituent' scarvacrol and thymol were low in the early phenological stage and increased gradually with plant development. The changes in the essential oil content (quantity and composition) varied for the studied plant species, according to corresponding environmental and growth factors and the major adaptive strategy toward summer drought that each plant species has evolved. Taking this into account, the harvest of these two species in order to obtain their essential oils offers acceptable economic possibilities (Müller-Riebau et al., 1997). Similar results were obtained as revealed the presence of different phenolic compounds like thymol and carvacrol which belong to terpenoids in both plant species (Marković et al., 2011).

Also, flavonoids presence in *Verbascum* species in general were recorded (Riaz et al., 2013). While, the phytochemical screening of *V. fruticulosum* in particular showed that aqueous and acetone extracts contained flavonoids. Total phenol and flavonoid contents were highest in acetone extracts. While, the methanol extract showed the highest total tannin content. So all extracts except n-hexane contained phenol and tannins (Fares et al., 2018).

The presence of such phytochemicals in the plant species under study may explain their effect on the attenuation of the coagulation cascade. This out finding coincides with what have been recorded in literature. For example, a study on the effect of phenol and certain phenyl compounds on the different stages of the blood coagulation and fibrinolytic mechanism was investigated. Phenol has a complex action on blood coagulation. It 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, the absence of factor XIII and calcium, it increased the solubility of fibrin in urea. Phenyl acetaldehyde, benzyl benzoate, and styrene (phenyl ethylene) accelerated clotting time and thrombin–fibrinogen reaction. Unpolymerized styrene and phenyl ethanol specifically inhibited the action of plasma thromboplastin, formed in a system lacking in factor VIII or IX. The contribution of platelets to plasma thromboplastin formation was examined in the light of these investigations, showing the ability of phosphorus-free compounds to supply a platelet-like activity (Nour-Eldin, 1968). *In vitro* platelets aggregation inhibition was referred to terpenoids which are phenols, explaining their extension tendencies to PT and aPPT (Cheng et al., 2014).

Moreover, since blood coagulation involves the generation of thrombin and conversion of fibrinogen into fibrin by active zymogens. This achieved via the activated factor X (FXa) which forms prothrombinase complex on phosphatidylserine containing surface by which prothrombin is converted to thrombin. One molecule of FXa generates more than 1000 thrombin molecules leading it to be a novel target for modern anticoagulant therapy. From this point of view the effects of the well-known plant polyphenolic compounds on factor Xa. Their results confirmed that only four polyphenols belonging to flavonoids group: procyanidin B2, cyanidin, quercetin and silybin, had inhibitory effect on FXa activity. This indicates that flavonoids might be potential structural bases for design of new nature-based, safe, orally bioavailable direct FXa inhibitors (Bijak et al., 2014). Moreover, the flavonoids and tannins proved their effect on the PT and aPPT (Adli et al., 2016), which went along with a haemostatic mechanism *in vivo* study that previously proved falvonoids effect on the coagulation cascade (Tanko et al., 2012).

Furthermore, the plants are known with their carbohydrates and proteins contents which were recorded to have bioactivity on the blood haemostasis. For example, polysaccharides such as pectins and hemicelluloses that were isolated from different plant species demonstrated antithrombin and thrombolytic activity (Mengome et al., 2014). Similarly, plant lectins affect blood haemostasis by increasing both PT and aPTT (Luz et al., 2013). In addition, cysteine proteases which are extracted from several plants latex exhibit fibrinolytic activity and reduce clotting time in a concentration dependent manner (Rajesh et al., 2007; Shivaprasad et al., 2009; Ramos et al., 2013).

As a result, plant extracts contain variable constituents which show different haemostatic bioactivity based on the type of extraction of a particular plant species. Therefore, this fact is confirmed by that the bioactivities of different plant species can be attributed to a synergic action among the molecules (Silva et al., 2009). So, the efficacy of the plant species extracts evaluation as anticoagulants or coagulants were related to various degree of solubility of different molecules. Nevertheless, the variability in the constitution of essential oils is also directly related to the species, and, within the same species. Since several factors can affect their composition, such as chemotype, location, collection period, and vegetative cycle (Gobbo-Neto and Lopes, 2007).

In conclusion, the positive recorded data would serve as a source of a novel, effective haemostatic agents that improve the management of cardiovascular diseases. In spite of that, further phytochemical studies for the purification and characterization of the active ingredients of the examined plant species extracts are still recommended.

Acknowledgements

We would like to thanks Biology and Biotechnology Department at An-Najah National University for allowing us to access their facilities.

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