

# The efficacy of Ankaferd Blood Stopper in antithrombotic drug-induced primary and secondary hemostatic abnormalities of a rat-bleeding model

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Ankaferd comprises a standardized mixture of plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum* and *Urtica dioica*. Ankaferd Blood Stopper (ABS) as a medicinal product has been approved in the management of external hemorrhage and dental surgery bleedings in Turkey. This study aimed to evaluate the in-vivo hemostatic effect of ABS in rats pretreated with acetylsalicylic acid or enoxaparin. Wistar rats (210–270 g) of both sexes were used in this study. The animals were pretreated with acetylsalicylic acid (10 mg/kg) orally for 4 days or enoxaparin sodium (8 mg/kg) subcutaneously for 3 days or did not receive any anticoagulant before tail cut at 4th day. ABS was administered topically [a total of 4 ml (1 ml/puff × 4)] to the cut tail in the studied animals. The duration of bleeding and the amount of bleeding were measured in order to evaluate the hemostatic effect of ABS. In acetylsalicylic acid-treated animals, topical ABS reduced both the duration and also the amount of bleeding volume by 68.4 and 54.6%, respectively. It was also effective in shortening the duration of bleeding (30.6%) and decreasing the amount of bleeding (32.8%) in enoxaparin-treated

animals. ABS, a traditional folkloric medicinal plant extract, has in-vivo hemostatic actions, which may provide a therapeutic potential for the management of patients with deficient hemostasis in the clinical medicine. *Blood Coagulation and Fibrinolysis* 20:185–190 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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## Introduction

Ankaferd is a medicinal plant extract, which has previously been used in Turkish traditional medicine as hemostatic agent [1]. Ankaferd comprises a standardized mixture of plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum* and *Urtica dioica*. Ankaferd Blood Stopper (ABS) as a medicinal product has been approved in the management of external hemorrhage and dental surgery bleedings in Turkey. The safety and efficacy reports on the product have indicated its sterility and nontoxicity.

A very recent in-vitro study by Goker *et al.* [1] has shown that exposure of ABS resulted in a very rapid formation of network within the plasma and serum. Routine hemostasis and biochemical tests revealed that the network formation due to ABS depended upon the interactions of the substance with the blood proteins, mainly fibrinogen, and indicated that ABS could affect both fibrinogen and other proteins possibly via agglutination of these molecules. The network of ABS might cover the entire physiological hemostatic process without affecting any

individual clotting factor [1]. Thus, this unique mechanism of action provides ABS with the advantage over other hemostatically active plant extracts and might, therefore, be effective both in individuals with normal hemostatic parameters and in those with primary, secondary or both hemostatic disorders.

In the light of the above-mentioned data, this study aimed to investigate the in-vivo hemostatic effect of ABS in rats. Additionally, the hemostatic effect of ABS was also evaluated in rats, which were pretreated with acetylsalicylic acid or enoxaparin.

## Animals and methods

### Animals

Forty-two Wistar albino rats (average weight 240 ± 30 g) of both sexes were used in this study. The animals were kept in a room at a constant temperature of 22 ± 1°C with a 12 h light and 12 h darkness cycle and fed standard pellet chow and water, which were available *ad libitum*. All experiments were carried out in accordance with the European Community Council

**Table 1 Description of study groups**

Group	Medication	Daily dose (mg/kg)	Route of administration	Duration	Solution applied to bleeding area
ASA-treated group/ABS	ASA	10	p.o.	4 days	ABS
ASA-treated group/control	ASA	10	p.o.	4 days	0.9% NaCl
Enoxaparin-treated group/ABS	Enoxaparin	8	s.c.	3 days	ABS
Enoxaparin-treated group/control	Enoxaparin	8	s.c.	3 days	0.9% NaCl
Untreated group/ABS	None	–	–	–	ABS
Untreated group/control	None	–	–	–	0.9% NaCl

ABS, Ankaferd Blood Stopper; ASA, acetylsalicylic acid, p.o., oral(ly); s.c., subcutaneous(ly).

Directive of 24 November 1986 (86/609/EEC) and were approved by the Fatih University Medical School Ethics Committee.

### Experimental design

The animals were divided into three groups for the tail-cut model (see Table 1).

First group ( $n = 14$ ) received 10 mg/kg oral acetylsalicylic acid (ASA) dissolved in isotonic saline by a feeding catheter custom-made of silver for 4 days (ASA group). At the end of the pretreatment period of 4 days, the tails of the rats were placed in a Plexiglass restraining device, prewarmed to 37°C in isotonic saline and cut transversely 4 cm from the tip. Tails of half of the animals ( $n = 7$ ) were treated with ABS topically [a total of 4 ml (four times 1 ml/puff)], whereas the other half ( $n = 7$ ) was treated with 4 ml isotonic saline (four times of 1 ml puff).

The animals in the second group ( $n = 14$ ) were administered with subcutaneous enoxaparin sodium at a dose of 8 mg/kg for 3 days (enoxaparin group). At the end of the pretreatment period of 3 days, the tail of the rats were placed in a Plexiglass restraining device, prewarmed to 37°C in isotonic saline, and cut transversely 4 cm from the tip. Tails of half of the animals ( $n = 7$ ) were treated with ABS topically [a total of 4 ml (four times 1 ml/puff)], whereas the other half ( $n = 7$ ) was treated with 4 ml isotonic saline (four times of 1 ml puff).

The animals in the third group ( $n = 14$ ) were not given any medication (control group). They served as the control group for the tail-cut model. Tails were cut transversely 4 cm from the tip. Tails of half of the animals ( $n = 7$ ) were treated with ABS topically [a total of 4 ml (four times 1 ml/puff)], whereas the other half ( $n = 7$ ) was treated with 4 ml isotonic saline (four times of 1 ml puff).

Before all operations, a list of randomization was developed by using RAND() function of Microsoft Excel. RAND() function generates random real numbers between 0 and 1. A table of two columns and 42 rows including  $2 \times 42$  cells was filled with RAND() function. Each row represented an individual rat. The numbers in the first column were used to determine the enrollment of

rats into three groups (ASA, enoxaparin and control groups). The random numbers in the first column were transformed to ranks from lowest to highest corresponding to 1–42. Rats ranked 1–14 were assigned to ASA group. Rats ranked as 15–28 and 29–42 were assigned to enoxaparin and control groups, respectively. Half of the rats in all groups were assigned to ABS and isotonic saline with a similar fashion of using random numbers. The random numbers in the second column were transformed to ranks from lowest to highest corresponding to 1–14, separately for all three groups. Rats ranked 1–7 were assigned to ABS group and rats ranked as 8–14 were assigned to control group.

Both ABS and isotonic saline were prepared in similar-looking dark-colored spray bottles earlier by a staff who worked neither in surgical operations nor in the evaluation of bleeding. The researchers and their assistants who had applied the study medication to the cut tails and who followed and evaluated the bleeding were not aware of the contents of the medication they applied, that is, they work blinded to the medication they used.

### Bleeding assay

Study parameters were duration of bleeding and amount of bleeding. Duration of bleeding was defined as the time passed after the start of bleeding (i.e. amputation or tail-cut) to cessation of bleeding. Bleeding start and stop times were measured by using a chronometer.

The amount of bleeding was measured by means of a blotting paper. The blood was collected on blotting paper, which was weighed before and after the procedure on a 0.1 g accurate scale. The difference in the weight of the blotting paper before and after the procedure indicated the amount of bleeding.

### Statistical analysis

The data were presented as mean and 95% confidence limits. Median and interquartile ranges (IQRs) were also given. In the initial analysis, data in each treatment group were analyzed separately with Mann–Whitney  $U$  test and also with independent samples Student's  $t$  test in order to test the robustness of data with regards to parametric assumptions.

**Table 2** The effect of topically administered Ankaferd Blood Stopper (4 ml) on the duration of bleeding of cut tails of rats pretreated with acetylsalicylic acid (10 mg/kg orally for 4 days), enoxaparin sodium (8 mg/kg subcutaneously for 3 days) and untreated rats

	ABS	Control	ABS vs. control	ABS vs. control, %	Statistics*	
Duration of bleeding (min)						
ASA treated	10.60 (7.46–13.74) (Median 12.00, IQR 6.00)	33.57 (28.03–39.12) (Median 34.00, IQR 11.00)	22.97 (17.29–28.64)	68.4	Z = 3.134 P = 0.001 <sup>a</sup>	t = 8.820 P < 0.001 <sup>b</sup>
Enoxaparin treated	44.00 (39.86–48.14) (Median 45.00, IQR 1.00)	63.43 (56.64–70.22) (Median 66.0, IQR 15.0)	19.43 (12.35–26.51)	30.6	Z = 3.148 P = 0.001 <sup>a</sup>	t = 5.977 P < 0.001 <sup>b</sup>
Untreated	1.81 (0.61–3.00) (Median 1.40, IQR 1.20)	25.61 (17.34–33.89) (Median 29.00, IQR 17.00)	23.81 (15.52–32.09)	92.9	Z = 3.134 P = 0.001 <sup>a</sup>	t = 6.966 P < 0.001 <sup>b</sup>
Amount of bleeding (ml)						
ASA treated	0.84 (0.41–1.28) (Median 0.70, IQR 0.50)	1.86 (1.48–2.23) (Median 1.90, IQR 0.40)	1.01 (0.50–1.53)	54.6	Z = 2.827 P = 0.002 <sup>a</sup>	t = 4.302 P = 0.001 <sup>b</sup>
Enoxaparin treated	2.20 (1.64–2.76) (Median 2.00, IQR 1.10)	3.27 (2.84–3.71) (Median 3.10, IQR 1.00)	1.07 (0.44–1.70)	32.8	Z = 2.380 P = 0.017 <sup>a</sup>	t = 3.704 P = 0.003 <sup>b</sup>
Untreated	0.62 (0.32–0.92) (Median 0.56, IQR 0.60)	1.60 (1.09–2.11) (Median 1.30, IQR 1.10)	0.98 (0.43–1.52)	61.1	Z = 2.820 P = 0.002 <sup>a</sup>	t = 4.023 P = 0.003 <sup>b</sup>

Data are given as mean (95% confidence interval) and median with IQR. ABS, Ankaferd Blood Stopper; ASA, acetylsalicylic acid; IQR, interquartile range. <sup>a</sup> Mann–Whitney *U* test. <sup>b</sup> Independent samples Student's *t* test. \*Statistical significance is set as *P* values less than 0.017 due to multiple pairwise comparisons (see section Statistical analysis).

As three comparisons (ABS vs. control in ASA-treated group, ABS vs. control in enoxaparin-treated group and ABS vs. control in untreated group) were performed instead of a single comparison for testing the primary hypothesis of the study 'whether the effect of ABS is significantly better than isotonic saline (whatever the animals are pretreated with)', the type I error level was adjusted downward to 0.017 (0.05 divided by three) for the results of Mann–Whitney *U* tests and independent samples Student's *t* tests. Therefore, *P* values less than 0.017 should be regarded as significant for these tests.

In addition to the univariate tests defined above, a two-way analysis of variance (ANOVA) model was built. In the model, which was fully saturated, the independent factors were the type of pretreatment (ASA vs. enoxaparin vs. untreated) and the type of solution applied to the cut tail (ABS vs. isotonic saline). The dependent variables were the duration of bleeding and the amount of bleeding for the first and second models, respectively. Therefore, the overall effects of ABS and anticoagulants used and their interaction were analyzed by two-way ANOVA. Overall effect of ABS corresponds to effect of ABS independent of the type of pretreatment (whether ASA treated, enoxaparin treated or untreated) and overall effect of anticoagulant treatment corresponds to effect of anticoagulant (whether treated with ABS or not). If ABS–anticoagulant interaction term is significant (*P* < 0.05), it means that the effect of ABS is not similar between anticoagulant-treated and untreated groups. Whenever the effect of anticoagulant is significant, pairwise comparisons were performed with *post hoc* Tukey's honestly significant difference (HSD) test. *P* values less than 0.05 were regarded as significant for two-way ANOVA and Tukey's HSD test. All analyses were done using SPSS version 9 statistical analysis package (SPSS, Inc., Chicago, Illinois, USA).

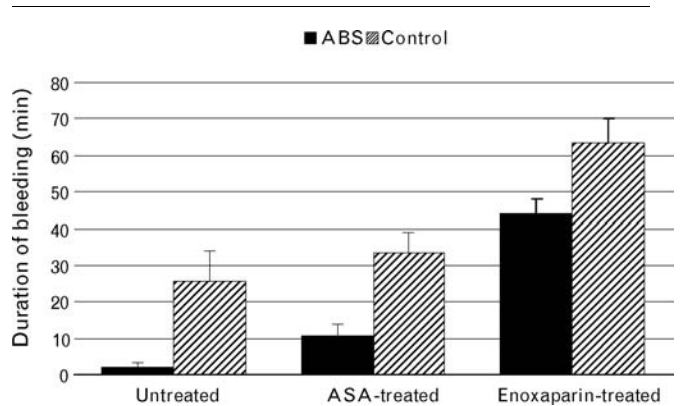
## Results

### Duration of bleeding

In the untreated group, duration of bleeding following tail cut was shortened by 23.81 min [95% confidence interval (CI) 15.52–32.09] with ABS administration from 25.61 min (95% CI 17.34–33.89) in the isotonic saline-administered control subgroup to 1.81 min (95% CI 0.61–3.00) (*P* = 0.001). ABS shortened the duration of bleeding by 92.9% in untreated group (Table 2, Fig. 1).

In the ASA-treated group, duration of bleeding following tail cut was shortened by 22.97 min (95% CI 17.29–28.64) with ABS administration from 33.57 min (95% CI 28.03–39.12) in the isotonic saline-administered control subgroup to 10.60 min (95% CI 7.46–13.74) (*P* = 0.001). ABS

**Fig. 1**



The effect of topically administered Ankaferd Blood Stopper (ABS, 4 ml) on the duration of bleeding of cut tails of rats pretreated with acetylsalicylic acid (ASA, 10 mg/kg orally for 4 days), enoxaparin sodium (8 mg/kg subcutaneously for 3 days) and untreated rats. Each group consisted of seven animals. Error bars correspond to 95% confidence interval. \**P* < 0.05 vs. control. CI, confidence interval.

**Table 3** The results of two-way analysis of variance with Ankaferd Blood Stopper effect and anticoagulant effect as independent factors and duration of bleeding and the amount of bleeding as dependent variables

Term in ANOVA	Duration of bleeding		Amount of bleeding	
	F <sup>a</sup>	P	F	P
ABS effect (ABS vs. control)	151.008	0.001	47.319	0.001
Anticoagulant effect (amongst anticoagulant groups)	184.032	0.001	46.562	0.001
ASA vs. control <sup>b</sup>		0.001		0.40
Enoxaparin vs. control <sup>b</sup>		0.001		0.001
ASA vs. enoxaparin <sup>b</sup>		0.001		0.001
ABS vs. anticoagulant interaction	0.558	0.58	0.034	0.97

ABS, Ankaferd Blood Stopper; ANOVA, analysis of variance; ASA, acetylsalicylic acid. <sup>a</sup>Two-way analysis of variance. <sup>b</sup>Tukey honestly significant difference (HSD) test.

shortened the duration of bleeding by 68.4% in ASA-treated group (Table 2, Fig. 1).

In the enoxaparin-treated group, duration of bleeding following tail cut was shortened by 19.43 min (95% CI 12.35–26.51) with ABS administration from 63.43 min (95% CI 56.64–70.22) in the isotonic saline-administered control subgroup to 44.00 min (95% CI 39.86–48.14) ( $P=0.001$ ). ABS shortened the duration of bleeding by 30.6% in enoxaparin-treated group (Table 2, Fig. 1).

ANOVA revealed that both the difference between ABS and isotonic saline subgroups (ABS effect) as well as the difference between ASA-treated, enoxaparin-treated and untreated groups (anticoagulant effect) were significant (ABS effect:  $F=151.008$ ,  $P<0.001$ ; anticoagulant effect:  $F=184.032$ ,  $P<0.001$ ). It was seen that the effect of ABS was similar among three study groups (ABS–anticoagulant interaction:  $F=0.558$ ,  $P=0.58$ ). In other words, ABS is significantly more effective in shortening the duration of bleeding as compared with control, and its effect is similar in all study groups (Table 3).

When the study groups were compared with Tukey's HSD test, duration of bleeding was different among all three groups. The difference in duration of bleeding between the ABS-treated and untreated groups was 8.38 min (95% CI 3.00–13.75,  $P=0.001$ ). This difference was more significant between the enoxaparin-treated and untreated groups, 40.00 min (95% CI 34.63–45.38,  $P<0.001$ ). When ASA-treated and enoxaparin-treated groups were compared, this difference was significant also [31.63 min (95% CI 26.25–37.00,  $P<0.001$ )].

#### Amount of bleeding

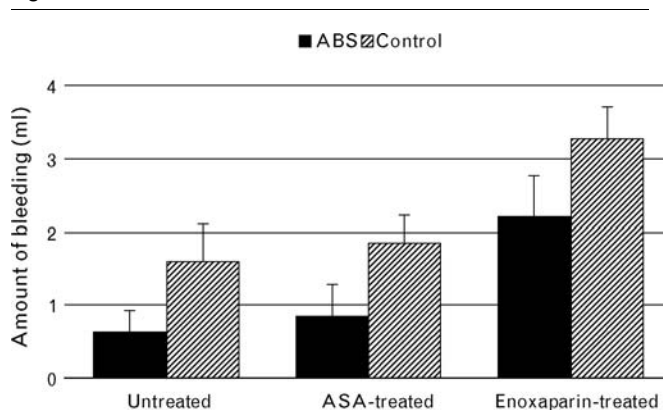
In the untreated group, the amount of bleeding following tail cut was decreased by 0.98 ml (95% CI 0.43–1.52) with ABS administration from 1.60 ml (95% CI 1.09–2.11) in the isotonic saline-administered control subgroup to 0.62 ml (95% CI 0.32–0.92) ( $P=0.002$ ). ABS decreased the amount of bleeding by 61.1% in untreated group (Table 2, Fig. 2).

In the ASA-treated group, the amount of bleeding following tail cut was decreased by 1.01 ml (95% CI 0.50–1.53)

with ABS administration from 1.86 ml (95% CI 1.48–2.23) in the isotonic saline-administered control subgroup to 0.84 ml (95% CI 0.41–1.28) ( $P=0.002$ ). ABS decreased the amount of bleeding by 54.6% in the ASA-treated group (Table 2, Fig. 2).

In the enoxaparin-treated group, the amount of bleeding following tail cut was decreased by 1.07 ml (95% CI 0.44–1.70) with ABS administration from 3.27 ml (95% CI 2.84–3.71) in the isotonic saline-administered control subgroup to 2.20 ml (95% CI 1.64–2.76) ( $P=0.017$ ). ABS decreased the amount of bleeding by 32.8% in the enoxaparin-treated group (Table 2, Fig. 2).

ANOVA revealed that both the difference between the ABS and isotonic saline subgroups (ABS effect) as well as the difference between ASA-treated, enoxaparin-treated and untreated groups (anticoagulant effect) were significant (ABS effect:  $F=47.319$ ,  $P<0.001$ ; anticoagulant effect:  $F=46.562$ ,  $P<0.001$ ). It was seen that the effect of ABS was similar among three study groups (ABS–anticoagulant interaction:  $F=0.034$ ,  $P=0.97$ ). In other words, ABS is significantly more effective in shortening

**Fig. 2**

The effect of topically administered Ankaferd Blood Stopper (ABS, 4 ml) on the amount of bleeding of cut tails of rats pretreated with acetylsalicylic acid (ASA, 10 mg/kg orally for 4 days), enoxaparin sodium (8 mg/kg, subcutaneously for 3 days) and untreated rats. Each group consisted of seven animals. Error bars correspond to 95% confidence interval. \* $P<0.05$  vs. control. CI, confidence interval.

the duration of bleeding as compared with control, and its effect is similar in all study groups (Table 3).

The study groups were compared pairwise by means of Tukey's HSD test. The difference in the amount of bleeding between the ABS-treated and untreated groups was not significant [0.24 ml (95% CI <0.00–0.68,  $P=0.40$ ). However, the amount of bleeding was significantly higher in the enoxaparin-treated group as compared with the untreated group [1.62 ml (95% CI 1.18–2.07,  $P<0.001$ ), and also with the ASA-treated group [1.39 ml (95% CI 0.94–1.83,  $P<0.001$ ).

## Discussion

The present in-vivo study on rats demonstrated that topically administered ABS has a hemostatic effect on rats alone or in the presence of aspirin or heparin. Thus, the present data support the recent in-vitro findings demonstrating the beneficial effect of ABS on hemostatic parameters [1]. Taken together, ABS seems to be a promising therapeutic agent for the management of clinically evident coagulation disorders.

The previous in-vitro study clearly demonstrated that addition of ABS to plasma did not affect the individual coagulation factors II, V, VII, VIII, IX, X, XI and XIII [1]. It decreased plasma fibrinogen activity concomitant with prolongation of the thrombin time [1]. Additionally, total protein, albumin and globulin levels showed significant decreases after addition of ABS. Thus, these results implied that the basic mechanism of ABS is the formation of an encapsulated protein network, which provides focal points for aggregation of red blood cells (RBCs). As ABS seems to provide a protein-driven agglutination without affecting the individual coagulation factors, it is advantageous over other plant extracts with hemostatic activity.

In the present study, we observed that ABS was beneficial as a topical hemostatic agent in bleeding rats. ABS was found effective in shortening the duration of bleeding and decreasing the bleeding volume in a tail-cut model. Interestingly, ABS showed its hemostatic action not only in untreated animals, but also in the animals pretreated with anticoagulants.

ASA and enoxaparin inhibit coagulation via different mechanisms. ASA or aspirin irreversibly blocks the formation of thromboxane-A<sub>2</sub> in platelets via inactivation of the cyclooxygenase enzyme and thus, produces an inhibitory effect of platelet aggregation. Heparin binding to the enzyme inhibitor anti-thrombin III (AT III) causes a conformational change resulting in its activation. The activated AT III then activates thrombin and other proteases involved in blood clotting, most notably factor X. ABS in rats treated with low-molecular-weight heparin (LMWH) is lower when compared with those treated with an antiplatelet drug. The difference between the

LMWH and aspirin could be due to both the unique mechanism of action of Ankaferd and the antihemostatic actions of both preparations. ABS represents its unique hemostatic effect by promoting the very rapid (<1 s) formation of a protein network, which acts as an anchor for vital physiological erythrocyte aggregation, covering the classical cascade model of the clotting system without independently acting on coagulation factors and platelets. As platelets are relatively spared during the Ankaferd action, ABS seems to be very effective to reverse antihemostatic action of ASA-induced hemorrhagic diathesis via the establishment of vital erythroid aggregation within a unique protein network to cover the defective platelets due to ASA administration. On the contrary, Ankaferd-induced hemostatic protein network interacts with thrombin upon its formation to form the anchor attracting RBCs. LMWH-induced anti-activated coagulation factor II effect might interfere with those pharmacobiological events in a negative way.

The data of the present study showed ABS was also effective in modulating hemostasis in rats pretreated with one of these conventional anticoagulants implying that application of ABS topically overcomes the actions of these agents given systemically. The beneficial action of ABS did not seem to be attenuated in animals, which pretreated with the anticoagulants, as confirmed by statistical analysis. Although the previous in-vitro study showed that ABS did not affect any of the individual clotting factors, we cannot exclude this possibility under in-vivo conditions. The duration of bleeding is known as an indicator of the effectiveness of platelet-thrombus formation, and therefore, a prolonged duration of bleeding may show the presence of severe thrombocytopenia, platelet dysfunction syndromes, vascular defects, mixed abnormalities such as von Willebrand's disease or all.

In the present study, shortening of the duration of bleeding by topical ABS suggests that the extract shows its hemostatic effect at least partly via modulation of the platelet functions. Other possible mechanisms of its action need further elucidation. Moreover, a very recent clinical case by Kurt *et al.* [2] presented a 52-year-old man who had undergone resection with the diagnosis of distal cholangiocarcinoma. He had signs of recent bleedings upon upper gastrointestinal endoscopic examination. Topical ABS (15 ml) showed effective control of bleeding from the biopsy site. A repeated endoscopy during the follow-up did not reveal any stigmata of bleeding as well.

ABS comprises a standardized mixture of five plants each having some hematological and vascular actions [3–8]. *Glycyrrhiza glabra* has anti-inflammatory, AT, antiplatelet, antioxidant, antiatherosclerotic and antitumor activities [6]. It inhibits angiogenesis, decreases vascular endothelial growth factor production and cytokine-induced neovascularization [6]. *Thymus vulgaris* has antioxidative actions

such as prevention of lipid peroxidation [4]. *Vitis vinifera* exerts antitumor and antiatherosclerotic effects [9,10]. *Alpinia officinarum* inhibits nitric oxide production by lipopolysaccharide-activated mouse peritoneal macrophages [3]. *Urtica dioica* causes vasodilation via inducing nitric oxide production by endothelium [5]. Thus, the mechanism(s) underlying the hemostatic control by ABS requires further investigation.

In conclusion, ABS, a traditional folkloric medicinal plant extract, may provide a therapeutic potential for the management of patients with deficient secondary hemostasis in the clinical medicine with its in-vivo hemostatic actions. The comparative, individual or both effects of Ankaferd on rats anticoagulated with warfarin would be an important topic in upcoming studies. In-vitro data on the antibacterial effects of Ankaferd [11] and preliminary successful applications in mediastinal bleedings associated with cardiac surgery [12] represent novel clues for Ankaferd activity. Phase I studies of ABS are completed, and Ankaferd is currently being studied in the treatment of Kırım-Kongo hemorrhagic fever with promising preliminary results, on the basis of its antiinfective and hemostatic efficacy [12–15] even with defective platelets, coagulation factors or both.

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