

Haemostatic Actions of the Folkloric Medicinal Plant Extract Ankaferd Blood Stopper®

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Ankaferd Blood Stopper® (ABS), a standardized mixture of five plants, has been used historically as a haemostatic agent but its mechanism of action remains unknown. This study investigated the *in vitro* effects of ABS on haemostatic parameters. When added to plasma or serum, ABS induced the very rapid formation of a protein network and erythrocyte aggregation. The levels of coagulation factors II, V, VII, VIII, IX, X, XI, and XIII were not affected by ABS. Plasma fibrinogen activity and antigen

levels were decreased following the addition of ABS, in parallel with the prolonged thrombin time. Total protein, albumin, and globulin levels decreased after the addition of ABS. Our findings suggest that ABS stimulates the formation of an encapsulated protein network that provides focal points for erythrocyte aggregation. ABS has the therapeutic potential to be used for the management of haemorrhage and this agent should be investigated further in clinical trials.

KEY WORDS: ANKAFERD BLOOD STOPPER® (ABS); *THYMUS VULGARIS*; *GLYCYRRHIZA GLABRA*; *VITIS VINIFERA*; *ALPINIA OFFICINARUM*; *URTICA DIOICA*; HERBAL REMEDIES; HAEMORRHAGE; HAEMOSTASIS

Introduction

Ankaferd Blood Stopper® (ABS) is a unique folkloric medicinal plant extract, which has historically been used in Turkish traditional medicine as a haemostatic agent. ABS comprises a standardized mixture of the plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum* and *Urtica dioica*. Each of these plants has some effect on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics and cell mediators,¹⁻⁶ however, the basic mechanism of action for the

haemostatic effects of ABS is currently unknown.

The aim of this study was to investigate the basic mechanism underlying the haemostatic actions of ABS. We studied the effect of ABS on the status of the principal primary and secondary haemostatic system components (e.g. coagulation proteins, platelets and blood cells) *in vitro* using routine haemostatic laboratory tests. Since haemorrhage can cause major morbidity and mortality, the identification of a novel, effective haemostatic agent might improve

the management of bleeding in a wide range of patients from all disciplines of clinical medicine.

Materials and methods

The study followed the procedures recommended by the following internationally accepted guidelines: EN ISO (International Organization for Standardization) 10993-1 'Biological evaluation of medical devices'; and EN ISO (International Organization for Standardization) 10993-4 'Biological evaluation of medical devices – Selection of tests for interactions with blood' (www.iso.org). This investigation was based on the principle of testing the effect of different ABS/plasma dilutions on distinct haemostatic parameters so should be considered as an hypothesis-generating descriptive study and, hence, no statistical tests were carried out.

ANKAFERD BLOOD STOPPER®

The sample of ABS (Ankaferd Blood Stopper®; patent number 2007-0-114485; Trend Teknoloji Ilaç AS, Istanbul, Turkey used in this study was lot number 0806002 (one vial of 100 ml).

HAEMATOLOGICAL TESTS

Ankaferd Blood Stopper® was diluted with pooled fresh normal human plasma in a range of dilutions from 0% to 50% in order to study the *in vitro* effects of ABS on several routine haematological parameters, including individual coagulation factors (II, V, VII, VIII, IX, X, XI and XIII), prothrombin time (international normalized ratio) (PT [INR]), activated partial thromboplastin time (aPTT), fibrinogen, thrombin time (TT), D-dimer test, platelet aggregation test and other haemostatic parameters. The dilutions were: 5% (1/20) comprising 50 µl

ABS/950 µl plasma, 10% (2/20) comprising 100 µl ABS/900 µl plasma, 15% (3/20) comprising 150 µl ABS/850 µl plasma, and so on up to a 50% dilution (10/20) comprising 500 µl ABS/500 µl plasma. All tests were carried out at 37°C and the dilutions were left for no longer than 15 min before carrying out the tests. Each test was repeated twice and the mean was calculated. Owren Koller buffer of approximately pH 7.35 was used for testing the fibrinogen and factor levels. Pooled plasma was used as controls. These tests were performed in the Haemostasis Laboratory, Department of Haematology, Hacettepe University Medical School, Ankara, Turkey using a STA-R Evolution® haemostasis device (Diagnostica Stago®, Asnières sur Seine, France) according to the manufacturer's instructions. Percentage coagulation factor activities were calculated from the calibration standards of the STA-R Evolution® haemostasis device.

MORPHOLOGICAL EVALUATIONS

Morphological evaluations and microscopic examinations of peripheral blood cells were carried out in the Morphology Laboratory, Department of Haematology, Hacettepe University Medical School and used an Olympus BX50 microscope attached with a Sony DSC-H5 camera. They were performed by adding 50 µl of ABS solution to a drop of fresh, whole human blood on a microscope slide.

BIOCHEMICAL TESTS

Ankaferd Blood Stopper® was diluted with pooled fresh normal human serum, and total protein, albumin and globulin levels were determined using routine biochemical methods in the Clinical Biochemistry Laboratory, Hacettepe University Medical School.

Results

Ankaferd Blood Stopper® induced very rapid (< 1 s) formation of a protein network in the plasma and serum samples (Fig. 1).

The levels of individual coagulation factors (II, V, VII, VIII, IX, X, XI, and XIII), PT (INR), aPTT, fibrinogen, TT, D-dimer, total protein, albumin and globulin after the addition of ABS to fresh normal plasma or serum are shown in Tables 1 and 2. Coagulation factors II, V, VII, VIII, IX, X, XI and XIII were not affected by the addition of ABS to plasma. Plasma fibrinogen activity decreased from 302 to > 10 mg/dl and fibrinogen antigen decreased from 299 mg/dl to < 30 mg/dl, in parallel with TT prolongation, following the addition of increasing proportions of ABS to pooled plasma.

Biochemical tests showed that total protein, albumin and globulin levels decreased after the addition of ABS to fresh serum (Table 2). Blood cells, particularly erythrocytes, were found to aggregate

rapidly (< 1 s) in the presence of ABS, thereby participating in protein network formation (Fig. 2). Hence, normal haemostatic elements were spared during formation of the protein network, the blood clotting process being driven by protein agglutination.

Discussion

In this study, we observed that the addition of ABS to normal plasma and serum resulted in the very rapid (< 1 s) formation of a protein network. We believe that this ABS-induced protein network was capable of regulating further coagulation and haemostatic reactions, however thromboelastographic studies intended to investigate the primary and secondary haemostatic components *in vitro* failed to proceed because of the presence of the ABS-induced protein network (data not shown).

Routine haemostatic and biochemical tests have revealed that the ABS-induced network formation depended upon

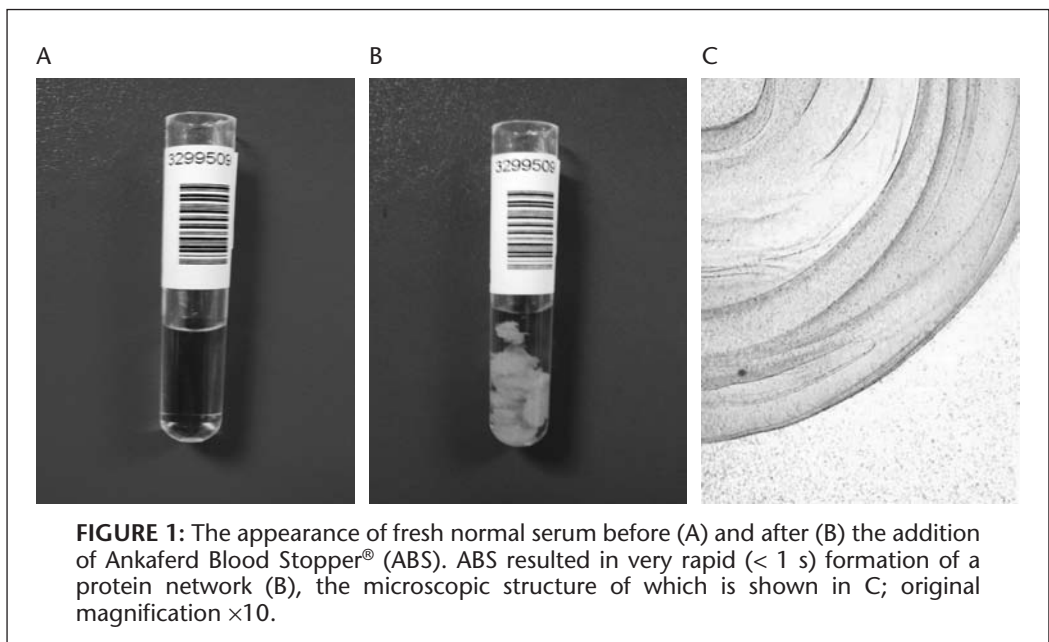


TABLE 1:
 Levels of individual coagulation factors (II, V, VII, VIII, IX, X, XI and XIII), PT (INR), aPTT, fibrinogen, D-dimer and thrombin time (TT) at various dilutions of Ankaferd Blood Stopper® (ABS) compared with controls

Sample	PT (INR)	aPTT (ratio)	Fibrinogen		Thrombin time (s)	D-dimer (µg/ml)	Coagulation factor activity (%) ^b												
			activity (mg/dl)	antigen (mg/dl)			II	V	VII	VIII	IX	X	XI	XIII urea clot lysis					
Pooled plasma basal sample	1.04	1.15	345	334	17.5														
Dilution ^a																			
1/20	1.40	1.60	302	299	20.8														
Control	1.06	1.15	324		17.5														
2/20	2.34	2.51	272	258	29.4														
Control	1.11	1.20	301		17.8														
3/20	4.35	4.26	226	170	71.7														
Control	1.14	1.17	298		17.2														
4/20	>65	>7.5	199	<30	>120	0.25	60	65	77	48	54	68	58						
Control	1.14	1.20	266		17.4	0.24	81	83	88	68	75	81	75						
5/20	>65	>7.5	123	<30	>120														
Control	1.20	1.18	244		17.3														
6/20	>65	>7.5	>10	<30	>120														
Control	1.27	1.22	215		18.1														
10/20																			
Control																			

^aTest' comprises dilutions of ABS/pooled plasma from 1/20 to 10/20. In the 'Control' the ABS was replaced by Owren Koller buffer.
^bPercentage coagulation factor activities were calculated from the calibration standards of the STA-R Evolution® haemostasis device that was used, according to the manufacturer's instructions.
 PT (INR), prothrombin time (international normalized ratio); aPTT, activated partial thromboplastin time.

TABLE 2:
 Levels of total protein, albumin and globulin at a 5/20 dilution of Ankaferd Blood Stopper® (ABS) with human serum compared with control

Sample	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
Pooled plasma basal sample	6.3	3.9	2.4
Dilution ^a			
5/20 Test	3.0	1.8	1.2
Control	5.5	3.5	2.0

^a'Test' comprises a dilution of ABS/pooled plasma of 5/20. In the 'Control' the ABS was replaced by Owren Koller buffer.

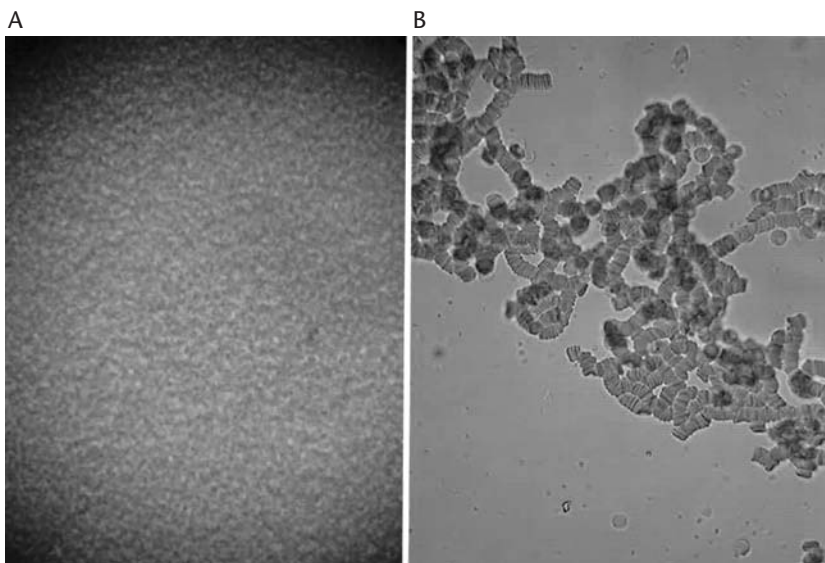


FIGURE 2: Light photomicrograph showing erythrocytes before (A; original magnification $\times 10$) and after (B; original magnification $\times 20$) the addition of Ankaferd Blood Stopper® (ABS) to fresh whole blood. ABS resulted in very rapid (< 1 s) aggregation of erythrocytes

interactions between ABS and blood proteins, mainly fibrinogen, and indicated that ABS could affect both fibrinogen and other proteins possibly via agglutination of these molecules. The ABS-induced network formation is related to the functions of blood proteins and red blood cells. The basic mechanism of action for ABS appears to be

the formation of an encapsulated protein network that provides focal points for erythrocyte aggregation. However, in plasma which contains fibrinogen, ABS also interacts with fibrinogen as well as other blood proteins. Since individual clotting factors (coagulation factors II, V, VII, VIII, IX, X, XI and XIII) were not affected by the

network formation, the anti-haemorrhagic process was possibly driven by protein agglutination. Blood cells (erythrocytes and platelets) also aggregated and participated in the network formation, with the erythrocytes forming a mass. These observations suggest that the ABS-induced formation of the protein network affected the entire physiological haemostatic process without unequally affecting any individual clotting factor. ABS might, therefore, be effective both in individuals with normal haemostatic parameters and in patients with deficient primary haemostasis and/or secondary haemostasis, including patients with disseminated intravascular coagulation. The basic mechanism of action for ABS appears to be the formation of an encapsulated protein network that provides focal points for erythrocyte aggregation. Exposure to ABS seems to provide a tissue oxygenation as well as a physiological haemostatic process without affecting any individual clotting factor. This unique mechanism of action provides ABS with an advantage over other haemostatically-active plant extracts.^{7,8}

Erythrocyte aggregation affects shear stress and microvascular flow dynamics.⁹ Some plant extracts can affect the normal rheological properties of erythrocytes and others penetrate erythrocyte membranes by modifying lipid-protein interactions.^{10,11} There are close relationships between protein concentrations, polymer type and erythrocyte aggregate formation.¹² Erythrocyte aggregation has also been observed in pathological states leading to hypercoagulability.⁹ Intensified erythrocyte aggregation locally produced in individual capillaries immediately disturbs normal blood flow inside the lumina and the rheological properties of blood flow in the microvessels.¹³ Erythrocytes also affect

platelet aggregation.¹⁴ Erythrocyte aggregation is determined by the concentration of high molecular weight plasma proteins, such as fibrinogen and immunoglobulins,¹⁵ and aggregation increases in the presence of fibrinogen.¹⁶ The possible involvement of erythrocyte hyperaggregation in the thrombotic process characteristic of thrombotic dysfibrinogenaemia has been shown.¹⁷ The dysfunctional fibrinogen molecule in thrombotic dysfibrinogenaemia influences the erythrocyte aggregation process to a greater extent than normal fibrinogen. Moreover, the dysfunctional fibrinogen molecule appears to have a stronger influence on the aggregation process than on plasma viscosity.¹⁸ Plasma cell disorders, such as multiple myeloma, represent 'disease models' for the interactions of neoplastic paraprotein, erythrocyte aggregation and TT prolongation. In our study, plasma fibrinogen activity and fibrinogen antigen levels decreased in parallel with TT prolongation after the addition of ABS. Likewise, biochemical tests also revealed that total protein, albumin, and globulin levels decreased after the addition of ABS. Hence, ABS seems to affect the fibrinogen-erythrocyte agglutination relationship, resulting in the formation of an encapsulated protein network that stimulates erythrocyte aggregation.

Ankaferd Blood Stopper® is a unique standardized mixture of the plants *T. vulgaris*, *G. glabra*, *V. vinifera*, *A. officinarum* and *U. dioica*, each of which has some effect on haematological and vascular parameters, and cellular proliferation.¹⁻⁶ For example, *G. glabra* inhibits angiogenesis, decreases vascular endothelial growth factor production and cytokine-induced neovascularization.⁴ *G. glabra* also has anti-inflammatory, anti-thrombin, anti-platelet,

anti-oxidant, anti-atherosclerotic, and anti-tumour activities.⁴ *T. vulgaris* has been shown to exhibit varying levels of anti-oxidant activity, which may help to prevent *in vivo* oxidative damage, such as lipid peroxidation, associated with atherosclerosis.² Inoculation experiments on detached leaves of *V. vinifera* exhibited enhanced resistance towards pathogens.^{5,6} *V. vinifera* also has anti-atherosclerotic and anti-tumour effects.^{19,20} *A. officinarum* inhibits nitric oxide (NO) production in lipopolysaccharide-activated mouse peritoneal macrophages.¹ *U. dioica* can produce hypotensive responses through a vasorelaxation effect mediated by the release of endothelial NO and the opening of potassium channels, and through a negative inotropic action.³ The combination of these plants in ABS appears to provide a unique composition for a tissue oxygenation and a physiological haemostatic process without disturbing the levels of any individual clotting factor. The effects of ABS on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular

dynamics and cellular mediators should be further investigated, however, in order to determine its potential role in many pathological states, including neoplastic disorders, infectious diseases, premature aging, atherosclerosis and diabetes.

Bleeding can cause significant morbidity and mortality in any clinical setting. ABS, a traditional folkloric medicinal plant extract, is a novel effective haemostatic agent that has the therapeutic potential to be used in the management of haemorrhage. It is hoped that clinical trials with this promising complimentary medicine will lead to the development of a new drug that is active in pathological haemostasis.

Acknowledgement

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Conflicts of interest

Ankaferd Blood Stopper® is a traditional folkloric medicinal plant extract that has been developed by HC Firat.

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