Research Article

Functional proteomic analysis of Ankaferd® Blood Stopper

Kanama durdurucu Ankaferd® ve etki mekanizmasının proteomik analizi

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Abstract

Objective: Ankaferd® Blood Stopper (ABS) comprises a standardized mixture of the plants Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum, and Urtica dioica. The basic mechanism of action for ABS is the formation of an encapsulated protein network that provides focal points for vital erythrocyte aggregation. ABS–induced protein network formation with blood cells, particularly erythrocytes, covers the primary and secondary hemostatic system without disturbing individual coagulation factors.

Materials and Methods: To understand the effect mechanisms of ABS on hemostasis, a proteomic analysis using 2D gel electrophoresis and mass spectrometer was performed.

Results: Proteins of plant origin in Ankaferd® were NADP-dependent-malic enzyme, ribulose bisphosphate-carboxylase-large chain, maturase K, ATP synthase subunit-beta, ATP synthase subunit-alpha, chalcone-flavanone isomerase-1, chalcone-flavanone isomerase-2, and actin-depolymerizing factor. Furthermore, functional proteomic studies revealed that proteins resembling human peptides have been detected within Ankaferd®, including ATP synthase, mucin-16 (CD164 sialomucin-like 2 protein), coiled-coil domain containing 141 hypothetical protein LOC283638 isoform 1, hypothetical protein LOC283638 isoform 2, dynactin 5, complex I intermediate-associated protein 30, mitochondrial, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, TP synthase, H+ transporting, mitochondrial actin binding 1 isoform, LIM domain and actin binding 1 isoform a, LIM domain and actin binding 1 isoform b, spectrin alpha non erythrocytic 1, prolactin releasing hormone receptor, utrophin, tet oncogene family member 2 isoform b, protein phosphatase 1 regulatory subunit 12A, NIMA (never in mitosis gene a)-related kinase, ATP-binding cassette protein C12, Homo sapiens malic enzyme 1, mitochondrial NADP(+)-dependent malic enzyme 3, ME2 protein, nuclear factor 1 B-type, abhydrolase domain-containing protein 12B, E3 SUMO-protein ligase PIAS2, alpha-1, 2-glucosyltransferase ALG10-A, cofilin, non-muscle isoform, 18 kDa phosphoprotein, p18, actin-depolymerizing factor (ADF), twinfilin-1, ankyrin repeat and FYVE domain-containing protein 1, usherin precursor, urotensin II receptor, interleukin 4, and midkine.

Conclusion: Proteomic analysis of Ankaferd® represents a true basis for the upcoming Ankaferd® studies focusing on its wound healing, hemostatic, anti-infective, antineoplastic, and preservative biological actions.

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Key words: Ankaferd, proteomics, hemostasis, bleeding

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Özet

Amaç: Kanama durdurucu olarak bilinen Ankaferd (AKD); Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum ve Urtica dioica bitki özlerinin özel bir karışımıdır. AKD nin temel etki mekanizması, hayati eritrosit agregasyon odakları olan enkapsüle protein ağı formasyonunu sağlayarak gerçekleşmektedir. AKD kan hücreleri ve özellikle eritrositler ile oluşumunu indüklediği protein ağı sayesinde birincil ve ikincil haemostatik sistem üzerine etkisini koagülasyon faktörlerini birebir hasarlamadan gerçekleştirir.

Yöntem ve Gereçler: ABS nin hemostaz üzerindeki etki mekanizmasını anlamak için 2D jel elektroforez ve kütle spektrometre yöntemleri kullanılarak proteomik analizleri yapıldı.

Bulgular: Ankaferd kanama durdurucu içeriğinde tanımlanan bitkisel proteinler: NADP-bağımlı malik enzim, Ribuloz bisfosfatkarboksilaz büyük zinciri, MturazK, ATPsentaz altünitesi beta, ATPsentaz altünitesi-alfa, Chalcon flavonon isomeraz-1, Chalcon-flavonon izomeraz 2 ve Aktin-depolimerizasyon faktördür. Ayrıca Ankaferd kapsamında koagülasyon için oldukça önemli farklı insan proteinleri benzerleri de tanımlanmıştır, bu proteinler arasında; ATP sentaz, musin16 (CD164-sialomucin-benzer-2 protein), helezonal kangal taşıyan protein-141, hypotetik protein LOC283638 izoform 1, hypothetik protein LOC283638 izoform 2, dinaktin 5, Kompleks 1 intermadia ilişikli protein 30, mitokondrial protein, NADH dehidrogenaz (Ubiquinone) 1 alpha altkompleks, TP sentaz H+ taşıyıcı protein, mitokondrial aktin bağlayıcı protein 1, LIM kangal ve aktin bağlayıcı alt ünite 1 izoform a, LIM kangal ve aktin bağlayıcı alt ünite 1 izoform b, Spectrin alpha non eritrotik 1, Prolactin releasing hormone reseptör, Utrophin, tet onkogen aile üyesi 2 izoform b, Protein fosfotaz 1 regulatory altünit 12A, NIMA -ilişkili kinaz, ATP-bağlayıcı protein C12, malik enzim 1, Mitochondrial NADP(+) bağımlı malik enzim 3, ME2 protein, Nuclear faktör 1B tipi , Abihidrolaz kangal taşıyıcı protein 12B, E3 SUMO-protein ligaz PIAS2, Alpha-1,2-glucosyltransferase ALG10-A, Cofilin, 18 kDa fosfoprotein, p18, Aktin-depolymerizing faktör, ADF, Twinfilin-1, Ankirin tekrarlayan ve FYVE kangalı içeren protein 1, Usherin öncü proteini, Urotensin II reseptör yer almaktadır.

Sonuç: Proteomik analizler sonucu elde edilen proteinler Ankaferdin hemostatik, yara iyileştirme ve anti-inflamatuvar etkilerinin araştırılmasına ışık tutacak ve açıklayıcı olacak niteliktedir. *(Turk J Hematol 2010; 27: 70-7)*

Anahtar kelimeler: Ankaferd, proteomiks, hemostaz, kanama

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Introduction

Ankaferd® Blood Stopper (ABS) is a standardized herbal extract obtained from five different plants: *Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum*, and *Urtica dioica* [1]. Topical safety of ABS in normal healthy human volunteers was shown in a randomized double-blind cross-over Phase I clinical trial after preclinical animal studies [2-6]. ABS has been approved for local topical applications in external post-surgical and post-dental surgery bleedings in Turkey (*www.Ankaferd.com*). Ankaferd has also been used topically for the management of hemorrhages uncontrolled by standard measures in a wide variety of difficult clinical conditions [7-13]. Ankaferd represents its unique local 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 [1,12]. Unique effects of critical proteins inside the ABS on critical transcription factors [14] and *in vitro* anti-infectious [15] and anti-cancer [16,17] effects suggested that Ankaferd may also affect pathobiological courses of tissues in addition to its unique action on hemostasis [18].

Functional proteomics is the essential step to identify the protein library of a given product, such as ABS hemostatic agent. The key characteristics of mass analyzers for proteomics are sensitivity, resolution and accuracy. Furthermore, demonstrated anti-infectious [15,19] and anti-neoplastic [16,17] actions of ABS *in vitro* represent the basis to search the *in vivo* efficacy and safety of this herbal product for rational phytotherapy in upcoming researches. Therefore, the protein content of ABS should be searched to proceed with those investigations. The aim of this study was to assess functional proteomics of ABS via matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analyses.

Materials and Methods

Two-Dimensional (2D) Sample Preparation

The protein samples for 2D gel electrophoresis were prepared as follows: 200 ml of Ankaferd solution was precipitated with trichloroacetic acid (TCA). 100 µl of 100% TCA was added for each 1 ml of sample after the vortex and ice bath incubation for 15 min and centrifuge at 14,000 *g* for 10 min at room temperature. The pellet was washed with 1 ml of ice-cold 85% acetone, vortexed to disperse pellet, then spun at top speed for 5 min at room temperature. The pellet was dried in a Speed Vac for 10-20 min to remove residual solvent. The pellet was re-suspended in 300 µl of 2D sample rehydration solution containing 7 M urea (Sigma, USA), 2M ThioUrea (Sigma, USA), 0.2% pH 3-10 linear IPG Ampholyte (Bio-Rad Laboratories, USA), 4% CHAPS (Sigma, USA), 1% HED (2-hydroxyethyldisulfide, Sigma, USA), and 1% DTT (Dithiothreitol, Sigma, USA). The total protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, USA).

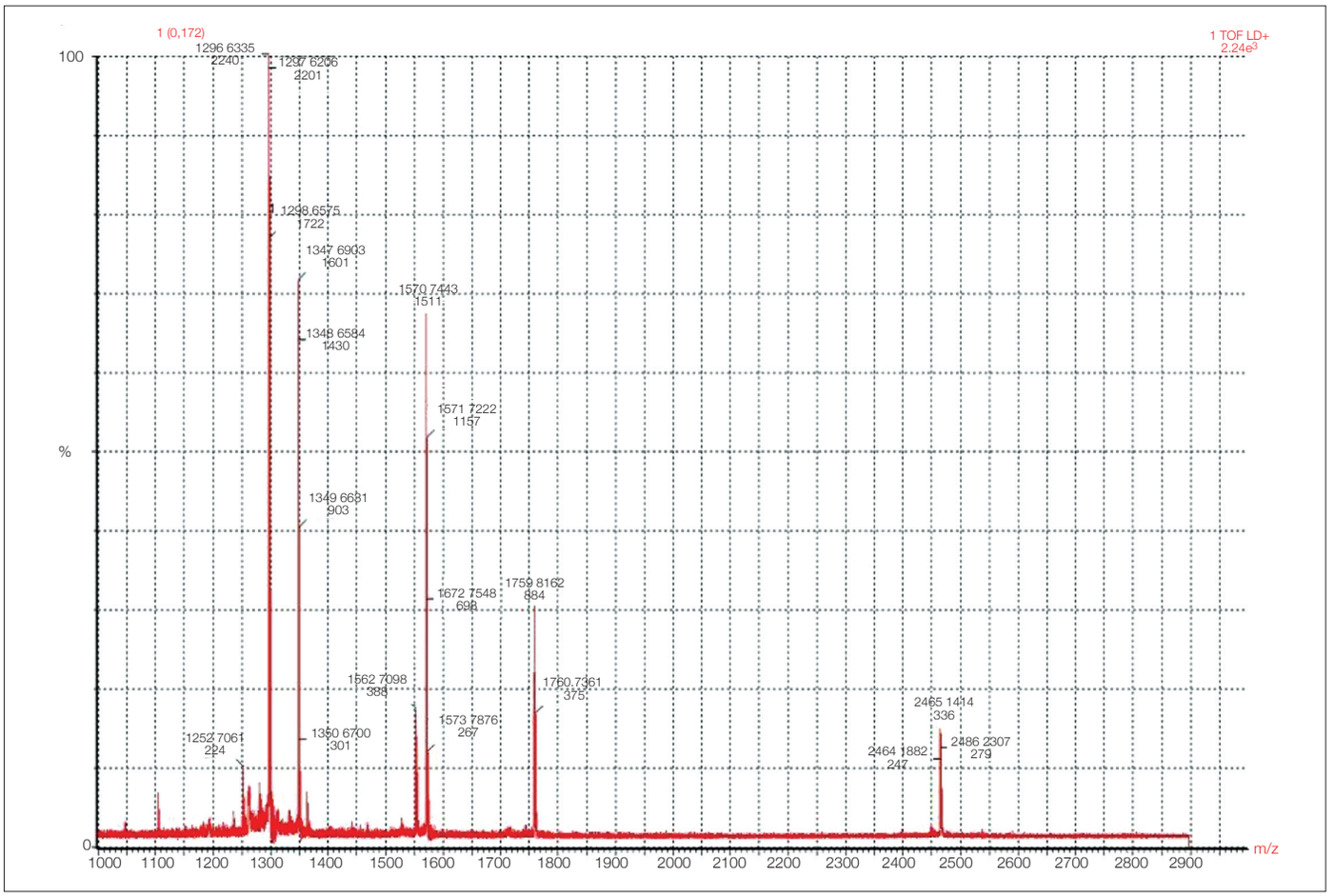

Figure 1. Spectra acquired from external calibration peptide mix, which includes sub-P, angiotensin, renin, ACTH and glu fib

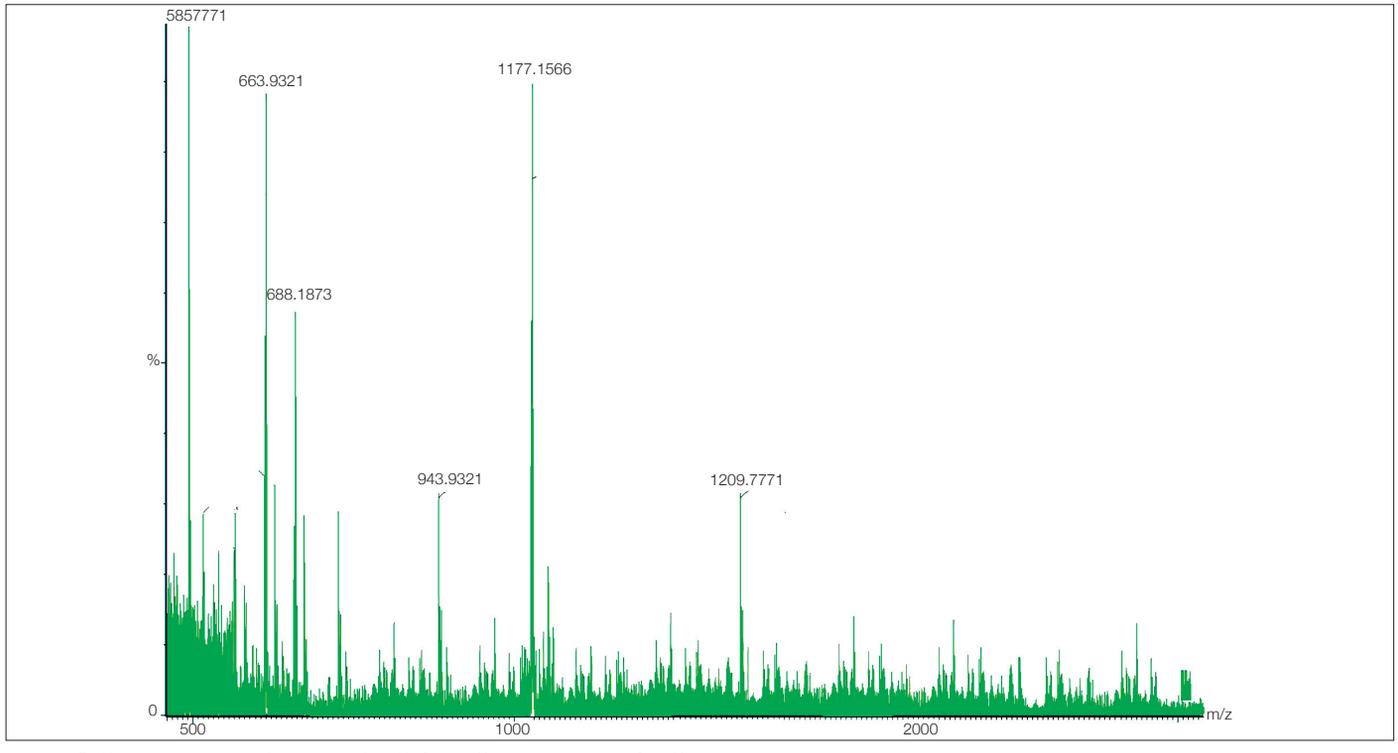

Figure 2. Spectra acquired from Ankaferd® Blood Stopper via MALDI-TOF

Two-Dimensional (2D) Gel Electrophoresis of Proteins

During the process, 150 µg of total proteins were rehydrated in 300 µl of rehydration buffer overnight (Bio-Rad Laboratories, USA). Samples were loaded on first dimension strips (17 cm, pI 3-10 Linear IPG). The first dimension of isoelectric focusing was performed using a Protein IEF cell (Bio-Rad Laboratories, USA). After running, first dimension gels were equilibrated for 10 min in equilibration buffer I (6 M urea, 0.375 M Tris pH 8.8, 2% SDS, 20% glycerol, 2% (W/V) DTT (Sigma, USA)), followed by 10 min in equilibration buffer II (6 M urea, 0.375 M Tris pH 8.8, 2% SDS, 20% glycerol, 2.5% (W/V) iodoacetamide). Samples were then separated by second dimension on 4-12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Gels were stained with Sypro Ruby protein stain, and images were acquired and analyzed using PDQuest software 8.0 (Bio-Rad Laboratories, USA).

Gel Digestion of Proteins

Selected spots from the gel were excised using a Proteome Works Spot Cutter (Bio-Rad Laboratories, USA) and transferred to a 96-well plate. The proteins were enzymatically digested and the tryptic peptides ZipTip (Millipore, France) purified. After ZipTip purification, the tryptic peptides were eluted from the ZipTip with 3 mg/100µl cyano-4-hydroxycinnamic acid (CHCA) solution in 50% acetonitrile (ACN)/0.1% TriFlora acetic acid and spotted directly onto wax-coated MALDI target plates.

Mass Spectrometry and Database Search

The tryptic peptides on the MALDI target plate were analyzed with MasLynx 4.0, MALDI-time of flight mass spectrometer (Waters, UK). Mass spectra were recorded in the positive-ion mode. All spectra were acquired with external calibration of sub-P, angiotensin, renin, ACTH and glu fib mix (Figure 1). PLGS (Waters, UK) was obtained with Swiss-Prot database with 50 ppm sensitivity. Proteins were evaluated by considering the number of matched tryptic peptides, the percentage coverage of the entire protein sequence, the apparent molecular weight (MW), and the pI of the protein (Figures 2, 3).

Results

To identify the hemostatic protein contents of ABS herbal extract, functional proteomic analysis was performed to shed light on the effect mechanisms of ABS on hemostasis. Dissolution of proteins was achieved by 2D gel electrophoresis. ABS, 1st dimension according to separation pI point and 2nd dimension separations according to MW, was evaluated by making a protein profile map with the help of fluorescent staining (Sypro-Ruby). PDQuest program 8.0 (Bio-Rad, USA) was used to analyze the protein profiles, and with the 3-D view analysis of the separated protein spots, the artifacts were eliminated and with the help of Proteome works, the spots were resumed from the gels (Figure 4). Proteins were digested

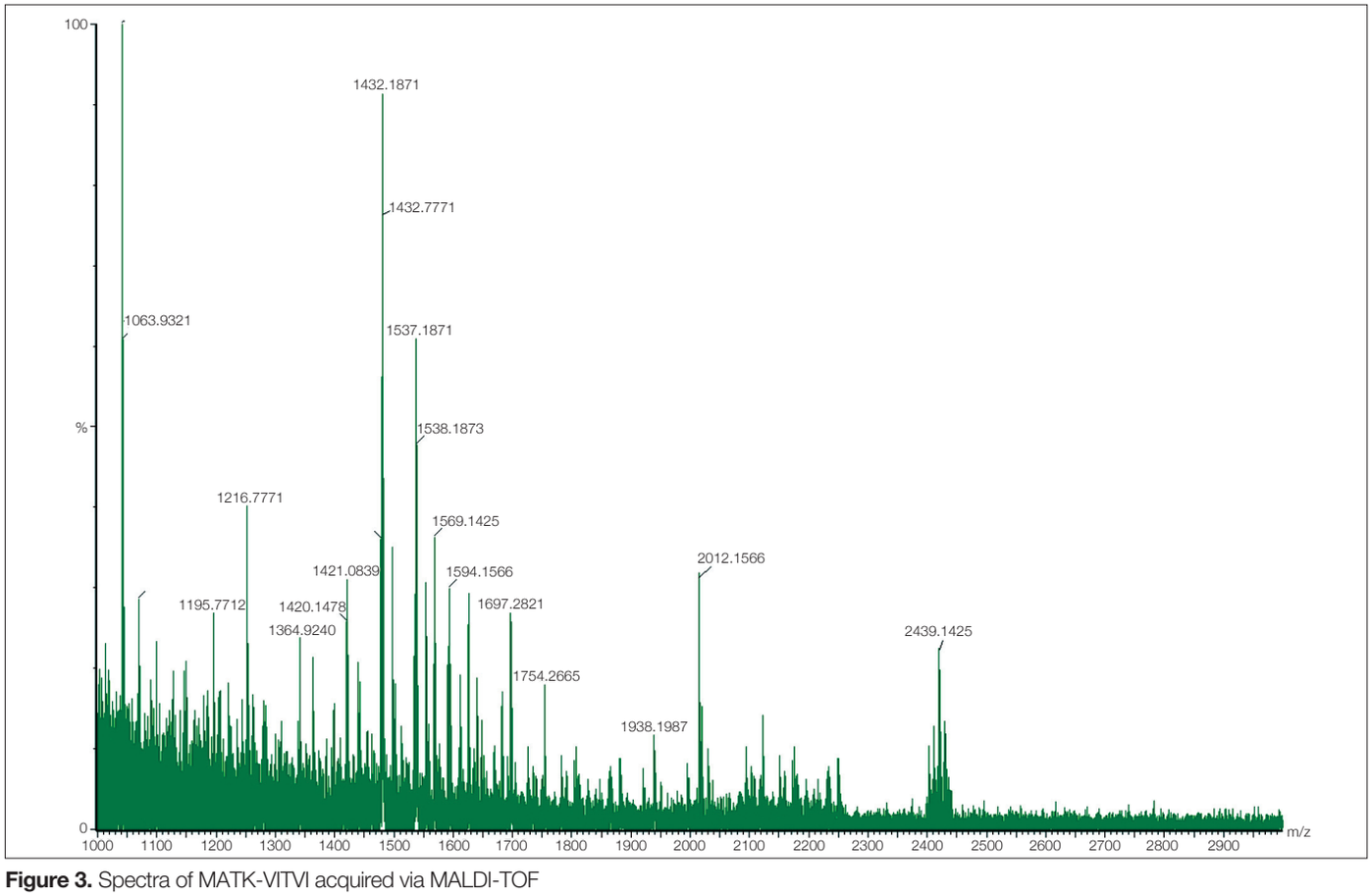

Figure 3. Spectra of MATK-VITVI acquired via MALDI-TOF

to peptides for mass spectrometer reflectron mode analysis in the range of 500-10.000 daltons.

In the protein description, MALDI-TOF mass spectrometry, which describes m/z values by calculating flight time by matrix-based laser-dependent ionization, was preferred. The ABS protein peptides were analyzed in positive ion mode with external calibration and with 50ppm sensitivity by MassLynx4.0 (Waters, UK). Moreover, the spectra data were identified with PLGS (Waters, UK) program using UniProt/ Swiss-Prot data banks. Peptide equalizations were re-checked and evaluated in accordance with MW and pI with 2D gel electrophoresis analyses. Furthermore, the herbal proteins and human-protein-like proteins were determined in ABS with the PLGS 5-7 PLGS score and 89-207 Mascot score.

Regarding the herbal analyses, the herbal proteins identified in ABS (Table 1) via 2D gel electrophoresis and mass spectrometer analysis with MALDI-TOF are:

- NADP-dependent malic enzyme
- Ribulose bisphosphate-carboxylase large chain
- Maturase K
- ATP synthase beta subunit
- ATP synthase alpha subunit
- Chalcone flavanone isomerase-1
- Chalcone flavanone isomerase 2
- Actin-depolymerization factor (ADF)

In addition, various human-protein-like proteins that are considerably important for hemostasis were also identified within ABS (Table 2). It was interesting to see that in addition to the herbal proteins, some human-like proteins that could represent many crossroads of hemostasis, infection, and neoplasia were found within ABS with the proteomic analysis.

Discussion

In this study, the protein library of Ankaferd has been evaluated via MALDI-TOF analyses. Topical hemostatic efficacy of ABS has been previously tested in animals with normal [2,5] and defective hemostasis [3,6]. Short-term hematological and biochemical safety of the oral systemic administration of Ankaferd to rabbits has been shown [20]. No acute mucosal toxicity, hematotoxicity, hepatotoxicity, nephrotoxicity, and biochemical toxicity was observed during the short-term follow-up of the animals [20]. Those preclinical results reflect a starting point to search any possible systemic confounding effect of ABS when applied to internal topical surfaces. Physiological cell-based coagulation could be clinically managed via topical ABS application to prevent and treat bleeding in many distinct clinicopathological states [7-13].

Ankaferd-induced formation of the protein network with vital erythroid aggregation covers the entire physiological hemostatic process. Mainly, there are distinct important components of the Ankaferd-induced hemostatic network. Vital erythroid aggregation takes place with the spectrin ankyrin and actin proteins on the membrane of red blood cells. Based on the results of our study, essential erythroid proteins (ankyrin recurrent and FYVE bundle containing protein 1, spectrin alpha, actin-depolymerization factor, actin-depolymerizing factor, LIM bundle and actin binding subunit 1 isoform a, LIM bundle and actin binding subunit 1 isoform b, NADP-dependent malic enzyme, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, mitochondrial NADP (+) dependent malic enzyme 3, ribulose bisphosphate- carboxylase large chain, maturase K) and the required ATP bioenergy (ATP synthase, ATP synthase beta subunit, ATP synthase alpha subunit, ATP-binding protein C12, TP synthase H+ transporter protein, ADF, and alpha-1,2-

Table 1. Plant proteins inside the Ankaferd®

Description	Accession No Uni ProtKB/Swiss-Prot	Theoretical pI/Mr	Measured pI/Mr
NADP-dependent malic enzyme	P51615	6/65.18	6.5/75
Rebulose bisphosphatem carboxylase large chain	Q37010	Undef/undef	9.0/100
Mt urase K	Q8HV93	9/62	7.0/55
ATP synthase subunit beta	Q0ZJ13	5.5/53	6.5/50
ATP synthase subunit alpha	Q0ZJ35	5.26/55,5	6.7/53
Chalcone-flavonone isomerase 1	P51117	5.25/25	6.5/35
Actin-depolymerzing factor	Q8SAG3	6.91/16.5	7/12

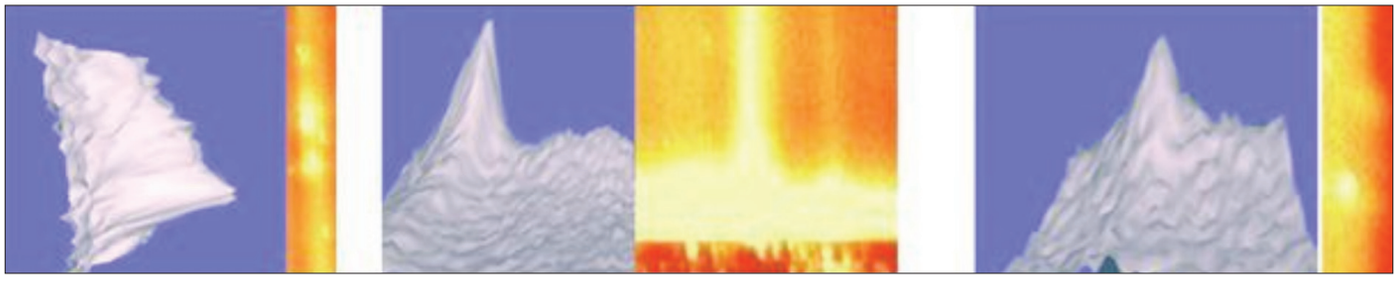

Figure 4. 3 Dimensional view of protein spots

Table 2. Proteins resembling human peptides in Ankaferd® protein library

TP synthase, H+ Transporting mitachondrial	A8K0K3	pl: 9.65/11400	8.00/25.000
acting binding 1 isoform	Q9UPN3	pl: 5.27/620.000	5.5/220.000
LIM domain and actin binding 1 isoform a			6.5/50.000
LIM domain and actin binding 1 isoform b			6.5/50.000
Spactirin alpha non erythrocytic 1	Q13813	pl: 5.22/28.400	6.5/40.000
Prolactin releasing hormone receptor	A5JUU5	pl: 9.43/41.100	9.00/40.000
Utroptin	P46939	pl: 5.21/40.000	6.5/40.000
tet oncogene family member 2 isoform b			6.5/50.000
Protein phosphatase 1 regulatory subunit 12A	O14974	pl: 5.31/115.200	6.5/120.000
NIMA never in mitosis gene a)- relatet kinas			
ATP binding cassette protein C12	Q96J65	pl: 8.64/152.000	7.00/150.000
highly similar to Homo sapiens malic enzyme 1	A8K168	pl: 5.79/64.100	6.5/75.000
Mitachondrial NADP (+) - dependent malic enzyme 3	Q6TCH8	pl: 6.31/29.700	6.5/30.000
Me2 protein	Q9BWL6	pl: 8.80/53.500	8.00/50.000
Nuclear factor 1 B-type	O00712	pl: 9.01/47.400	9.00/50.000
Abhydrolase domain-containing protein 12 B	Q7Z5M8	pl: 8.57/40.000	8.5/50.000
E3 SUMO-protein ligase PIAS2	O75928	pl: 7.47/68.200	7.5/75.000
Alpha- 1,2-glucosyltransferase ALG10-A	Q5BKT4	pl: 9.40/55.600	9.5/70.000
Cofilin, non-muscle isoform			
18 kDa phosphoprotein			
p18	P23528	pl: 8.26/18.500	8.5/35.000
Actin-depolymerizing factor			
ADF	P60981	pl: 8.12/18.500	8.00/30.000
Twinfilin-1	Q12792	pl: 5.7/128.200	6,5/120.000
Ankyrin repeat and repeat and FYVE domain containing protein 1	Q9P2R3	pl: 6.47/42.201	7.00/50.000
Usherin [Precursor]	O75445	pl: 6.42/572.200	6.5/220.000
Urotensin II receptor	Q9UKP6	pl: 10.6/42.100	9.57/50.000
IL-4 receptor			
IL-4ligand			
Midkine			

glycosyltransferase ALG10-A) are included in the protein library of Ankaferd. Ankaferd also upregulates GATA/FOG transcription system affecting erythroid functions and urotensin II [14,18]. Urotensin II is also an essential component of Ankaferd and represents the link between injured vascular endothelium, adhesive proteins and active erythroid cells. Those concepts could be developed via MALDI-TOF proteomic molecular analyses, cytometric arrays, transcription analysis, and Scanning electron microscope (SEM) ultrastructural examinations as well as upcoming investigations interacting with basic and clinical research facilities.

Ankaferd, in addition to its hemostatic activity, may also inhibit the growth of bacteria [19]. The anti-infectious activity of Ankaferd may represent an advantage over its current clinical use, since it inhibits the growth of bacteria in the area used mainly for its hemostatic activity, such as in traumatic infected wounds. The antimicrobial activity of Ankaferd was tested against many pathogens [15]. The isolates included *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter spp., Stenotrophomonas maltophilia,* methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant coagulase negative *Staphylococcus,* vancomycin-susceptible *Enterococcus,* and vancomycin-resistant enterococci (VRE). Antibacterial activities of Ankaferd against several gram-positive and gram-negative food and human pathogens were also reported in another study [19]. The mechanism of action regarding the anti-infective activity of ABS is currently unknown. Several proteins (Homo sapiens malic enzyme 1, dynactin 5, cofilin, utrophin, mucin 16 (CD164-sialomucin-

like-2 protein), chalcone flavanone isomerase-1, chalcone flavanone isomerase 2, helezonal bundle transporter protein-141, hypothetical protein LOC283638 isoform 1, hypothetical protein LOC283638 isoform 2, complex I intermediate-related protein 30) in our proteomic analyses represent an important step to elucidate how Ankaferd biologically affects the components of numerous pathogens.

Several hypotheses will need to be raised to understand the mechanism-of-action of Ankaferd on tumor tissue [16,17]. ABS is a hemostatic agent with pleiotropic effects [14,18,21,22]. The unique protein library of Ankaferd upregulates critical transcription factors including regulators of neoplasia such as p53 [14,18]. There is a close relationship between coagulation factor expressions and solid tumor progression, via mechanisms other than angiogenesis. Several proteins (midkine, interleukin 4, p18, 18 kDa phosphoprotein, SUMO-protein ligase PIAS2, abhydrolase bundle transporter protein, the precursor of usher, tet oncogene family member 2 isoform b, twinfilin-1, SUMO-protein ligase PIAS2, prolactin secretor hormone receptor, protein phosphatase 1 regulatory subunit 12A, never in mitosis gene a (NIMA)-related kinase, mitochondrial protein, mitochondrial actin binding protein 1) in our proteomic analyses represent an important step to elucidate how Ankaferd regulates cell cycle and other biological actions of the tumor tissue.

The pleiotropic effects of ABS on vascular endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics, and cellular mediators should be investigated to determine its potential role in many pathological states, including neoplastic disorders, infectious diseases and inflammation. Our proteomic results in this report within many crossroads of hemostasis, infection and neoplasia may shed further light and represent a novel starting point on that perspective for the new avenues of ABS.

Acknowledgements

There are no conflicts of interest to report.

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