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Proteomic and transcriptomic analyses to explain the pleiotropic effects of Ankaferd blood stopper

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Abstract

Ankaferd blood stopper is a standardized mixture of the plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica* and has been used as a topical hemostatic agent and with its clinical application established in randomized controlled trials and case reports. Ankaferd has been successfully used in gastrointestinal endobronchial mucosal and cutaneous bleedings and also in abdominal, thoracic, dental and oropharyngeal, and pelvic surgeries. Ankaferd's hemostatic action is thought to form a protein complex with coagulation factors that facilitate adhesion of blood components. Besides its hemostatic action, Ankaferd has demonstrated pleiotropic effects, including anti-neoplastic and anti-microbial activities and tissue-healing properties; the underlying mechanisms for these have not been well studied. Ankaferd's individual components were determined by proteomic and chemical analyses. Ankaferd also augments transcription of some transcription factors which is shown with transcriptomic analysis. The independent effects of these ingredients and augmented transcription factors are not known precisely. Here, we review what is known of Ankaferd blood stopper components from chemical, proteomic, and transcriptomic analyses and propose that individual components can explain some pleiotropic effects of Ankaferd. Certainly more research is needed focusing on individual ingredients of Ankaferd to elucidate their precise and effects.

Keywords

Ankaferd blood stopper, hemostasis, proteomic analysis, genomic analysis, pleiotropic effect

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Ankaferd Blood Stopper is a standardized mixture of plants Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia offacinarum and Urticora dioica plants and have been used as a topical hemostatic agent. As a traditional medicine, Ankaferd is shown to have pleiotropic effects. ABS demonstrated anti-neoplastic, anti-microbial actions, also promoted tissue healing. In this study, literature about ingredients of ABS formula with respect to previously performed chemical, transcriptomic and genomic analysis is presented to explain these pleiotropic effects.

Introduction and effects of Ankaferd

Ankaferd is a novel hemostatic agent that was formulated from a traditional extract used in Anatolia and is a standardized mixture of the plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*. Ankaferd's hemostatic action and clinical application has been established in randomized controlled trials and case reports. However, Ankaferd has pleiotropic effects including anti-neoplastic, anti-microbial, anti-mutagenic, and antioxidant as well as tissue-healing properties,[1] although the underlying mechanisms have not been well studied. Here, we review the literature on Ankaferd to clarify the mechanistic basis of this pleiotropism. The procoagulant function of Ankaferd blood stopper (ABS) has been extensively discussed elsewhere.[2]

Ankaferd has been successfully used as a topical hemostatic agent, for example, in the treatment of digestive tract ulcers as well as various types of bleeding including variceal/non-variceal, gastrointestinal,[3–5] endobronchial,[6] mucosal (including in patients with hemorrhagic diathesis[7–9]), and cutaneous.[10] Ankaferd has also been employed in abdominal, thoracic, dental and oropharyngeal, and pelvic surgeries (Table 1).[11–14] The precise mechanism of Ankaferd's

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Table 1. Clinical trials on Ankareferd's hemostatic action.

Author	Year	Number of population	Study design	Diagnosis of patients	Clinical settings	Conclusions	Perspectives
Yaman et al.[15]	2012	30	Randomized	Carious primary molar teeth of 6–9 years old children	ABS was compared with formocresol for 3, 6, and 12 months for pain, swelling, mobility, resorption, furcation, and periapical bone destruction	ABS was as effective as formocresol as a pulp dressing of primary molar	ABS appears to be an alternative pulpectomy agent
Iynen et al.[16]	2011	90	Prospective	<18-year-old patients who needed adenoidectomy	The study assessed the clinical effect of ABS on hemostasis in adenoidectomy and post-adenoidectomy patients	ABS reduces the duration and blood loss of an adenoidectomy and increases postoperative quality of life	In situations when pinpointing the source of the hemorrhage in the nasopharynx is difficult, tampons soaked in ABS are extremely useful in stopping the hemorrhage
Teker et al.[17]	2009	49	Prospective, randomized, controlled, non-blinded, clinical trial	Patients with anterior epistaxis	The efficacy of ABS as a hemostatic agent compared to hemostasis by phenylephrine was observed	ABS is effective, safe, quick, and easy alternative to the phenylephrine in patients with anterior epistaxis	ABS is an effective agent to control bleeding in cases that could not be managed by vasoconstrictor agents
Uzun et al. [6]	2013	20	Retrospective study	Patients with hemoptysis who needed bronchoscopic procedures	The study was done to evaluate the hemostatic efficacy of endobronchial application of ABS solution in patients with hemoptysis	Bleeding could be controlled with ABS a few seconds after instillation in the hemorrhagic focus in 23 interventions, but it was ineffective in 2 cases.	Bronchoscopic application of ABS may be an alternative supportive treatment in cases of uncontrolled hemoptysis.
Guler et al.[18]	2011	61	Prospective, randomized, controlled trial	Patients with benign euthyroid multinodular goiter who needed total thyroidectomy	The study was designed prospectively to compare ABS and HCT groups in terms of operation time, postoperative drainage, duration of postoperative stay, and complications	The use of ABS is more effective than HCT to control hemorrhage following total thyroidectomy	The usage of ABS might be more effective in preventing hematoma by stopping oozing-type bleeding without causing an increase in hemostasis-related complications
Pamuk et al.[20]	2015	15	Prospective, randomized clinical study	Patients with chronic periodontitis	Following the initial periodontal therapy, patients were randomly assigned to two treatments in contralateral areas of the dentition: ACB + ABS or ACB alone and the procedures were applied	ABS enhances the soft tissue healing during the periodontal defect fill by the ACB by stimulating angiogenesis and vascular endothelial cell function, prevents GR and thereby increases the clinical attachment gain	The results indicate that (1) both treatment modalities resulted in statistically significant clinical improvements compared with baseline, (2) ABS may improve the regenerative process and cause less GR, and (3) ABS may lead to an increase in levels of the VEGF in the healing stage of periodontal surgery
Istanbulluoglu et al.[21]	2013	90	Prospective, randomized clinical study	Patients who undergone PCNL because of renal and/or upper ureter stones	45 of the patients underwent tubeless PCNL with the use of ABS as a hemostatic agent, whereas the remaining ones underwent tubeless PCNL without ABS and the procedures were applied	ABS is an efficient and reliable hemostatic agent in tubeless PCNL	ABS is a safe and reliable method in tubeless or totally tubeless PCNL interventions leading to expectations that these procedures might find widespread use among endourologists
Amer et al.[22]	2013	205	Prospective, randomized clinical study	Patients with a single tooth to be extracted that can be removed with forceps without the need for mucoperiosteal flap and/or dental elevators were included in this study	Patients were selected so that 80 patients have INR values of ≤2, whereas the remaining patients have the INR values ranging from 2 to 3 and the procedures were applied	ABS is an effective hemostatic agent comparable to tranexamic acid in controlling post-extraction bleeding in AOT patients of INR values ≤3 with no evidence support the superiority of tranexamic acid over ABS	For patients with INR values >2 ABS can represent itself as a sufficient local hemostatic agent that is comparable with tranexamic acid

Table 1. (Continued)

Author	Year	Number of population	Study design	Diagnosis of patients	Clinical settings	Conclusions	Perspectives
Atalay et al.[23]	2015	50	Randomized, prospective clinical study	CABG patients who medicated with clopidogrel and ASA prior to CABG surgery	25 CABG patients received a high-dose clopidogrel (600 mg) and 300 mg ASA have been included into the study (ABC group). 25 patients have also been included into the study for comparison and the procedures were applied	Local use of ABC decrease the bleeding from the mediastinum after CABG	ABC seems to be effective agent to inhibit blood loss after CABG without any complication and provides a significant reduction of bleeding in patients medicated with high-dose clopidogrel
Yasar et al.[24]	2009	60	Prospective, non-randomized, non-blinded observational study	Patients who were subjected to the intended procedure if having an upper airway obstruction due to adenoid tissue	Each child was assigned either to the ABS or the SS group in order of appearance on the surgical waiting list	A statistically significantly shorter duration of bleeding and a statistically lower number of packs are required to achieve ABS tamponade-induced hemostasis during adenoidectomy as compared to saline soaked gauze sponge application	ABS aids in the control of intraoperative bleeding and reduces the number of packs required to achieve hemostasis, so that it can be recommended for tamponades performed during pediatric adenoidectomies
Teker et al.[17]	2009	47	Prospective, non-randomized, non-blinded study	Patients with chronic tonsillitis, tonsillar hypertrophy, and obstructive sleep apnea syndrome	Patients with bleeding disorders, aspirin use within 2 weeks prior to surgery, peritonsillar abscess history, acute tonsillitis within 4 weeks prior to surgery, tonsillectomy due to malignity suspicion, and children with systemic diseases were excluded and the procedures were applied	ABS reduces intraoperative hemorrhage and operation time	It is a safe, efficient, and easy to use hemostatic agent with no side effects and it is recommended to use ABS during routine tonsillectomy for healthy children
Akpinar et al.[19]	2015	50	Double-blind, placebo-controlled, randomized clinical trial	Patients with unstable angina unsuitable for percutaneous coronary intervention who were scheduled for urgent or acute CABG	Twenty-five emergency CABG patients premedicated with clopidogrel and ASA were included in the study (Group 1). An additional 25 patients who were premedicated with the same antiplatelet agents were selected as a control group (Group 2)	The use of local ABS reduces bleeding, transfusion requirements of packed red blood cells, platelets, and total blood units in patients premedicated with clopidogrel and ASA undergoing emergent CABG	This study demonstrated that a significant reduction in bleeding and requirement for transfusion can be achieved with the use of ABS in emergency CABG patients premedicated with a high dose of clopidogrel
Eyi Yapar et al. [25]	2012	40	Prospective, randomized clinical study	Pregnant women with a term singleton fetus in a vertex position who required a mediolateral episiotomy	The patients were randomly assigned to two approaches (20 to ABS, 20 to SS)	Application of 4 mL of ABS instead of SS lessened bleeding	The study revealed a positive effect of the topical application of ABS tested for bleeding reduction
Atay et al.[9]	2013	20	Prospective, randomized clinical study	Patients with oral mucositis of grade 3–4 according to the WHO classification	After patients developed oral mucositis they used only ABS, and age and gender of patients, type of the underlying malignant disease and used chemotherapeutic drugs, frequency, amount and duration of ABS use and healing time of oral mucositis were recorded	The healing duration of oral mucositis was shorter with the topical ABS application. And also the hemorrhages from oral mucositis lesions were recovered within 2 days with ABS	ABS is an effective agent in the chemotherapy-related severe oral mucositis treatment of the patients with hematological malignancies. ABS shortens the healing time with acceptable side effects
[26]	2008	23	Clinical trial	Dental treatments with bleeding such as periodontitis, tooth removal, etc.	Use of ABS in dental procedures was observed both by physical examination and laboratory tests	Laboratory findings were not affected by ABS application. No GIS side effects were observed	ABS is safe for oral-topical use

ACB: autogenous cortical bone greft, ABS: Ankaferd blood stopper; AOT: Oral anticoagulant therapy; CABG: coronary artery bypass grafting; ASA: acetylsalicylic acid; INR: international normalized ratio; GIS: gastrointestinal system; GR: gingival recession; HCT : hemostasis by conventional technique; PCNL: percutaneous nephrolithotomy; SS: saline solution; VEGF: vascular endothelial growth factor.

hemostatic action is not known, but it is thought to form a protein complex with intrinsic coagulation factors that facilitate erythrocyte and thrombocyte adhesion. The hemostatic mechanism of Ankaferd will not be further addressed here; instead, we refer readers to a recent review of this subject.[2]

Ankaferd has long been known to promote the repair of bone, periodontal, muscle, skin, gastrointestinal, and oropharyngeal tissues and urogenital mucosae after surgery or injury, although some contradictory findings have been reported.[27–30]

The antimicrobial activity of Ankaferd is thought to derive from its oxygen-enhancing capacity through erythrocyte aggregation. The antimicrobial spectrum is broad and includes microorganisms such as methicillin-resistant *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella, Acinetobacter baumannii, Mycobacterium tuberculosis*, and *Candida albicans*.[31,32] Ankaferd has also been used to treat hydatid cysts via injection.[33,34]

Less is known about the anti-neoplastic activity of Ankaferd, although it was shown to inhibit cell proliferation, promote apoptosis, and prevent blast cell transformation of B-cell chronic lymphocytic leukemia cells in vitro.[35] It has also demonstrated toxicity toward multiple myeloma and plasmacytoma cells both in vitro and in vivo, while decreasing M protein production.[36,37] Other solid malignancies such as sarcoma and colon cancer are also targets of the anti-neoplastic action of Ankaferd.

Method

The constituents of Ankaferd have been identified by proteomic and transcriptomic analyses.[38,39] Proteins were searched in the Uniprot Protein Knowledge Database by accession number, whereas genes were searched in the GeneCards database. Both proteins and genes were searched in National Library for Health and Institute for Scientific Information databases. References and related literature were reviewed by three authors. Based on this information, we hypothesized that the constituents of Ankaferd contribute to each of its three major pleiotropic actions, which are discussed below.

Antimicrobial effects

Ankaferd has antibacterial, germicidal, and antimycobacterial effects. In vitro studies have shown that ABS is effective against both Gram-positive and Gram-negative bacteria;[40] foodborne pathogens such as *Escherichia coli* and *Salmonella typhimurium*;[41] resistant nosocomial pathogens such as *Pseudomonas*, *Klebsiella*, *Acinetobacter*, *Enterococcus*, and *Staphylococcus* species;[42] *Echinococcus granulosus*;[33] and resistant *Tuberculosis* strains.

The antimicrobial effects of Ankaferd may be attributable to its chemical components. A time-of-flight mass spectroscopy analysis of ABS revealed the presence of the antioxidants tocotrienol, tryptophan, thymol, lycophene, enoxolone, tomatine, tertiary butylhydroquinone, vitamin E derivatives, and galangin.[43]

Anti-neoplastic effects

Another property of Ankaferd is the capacity to inhibit neoplasia. ABS has demonstrated cytotoxicity against human erythrocytes at some concentrations, as well as against tumoral cells in hematologic malignancies such as multiple myeloma, chronic myelogenous leukemia, and lymphoma.[35,37,44] Possible related ingredients of Ankaferd are CREBZF, PIAS-2, HNF-4a, ME-1, P18INK4C, and Midkine and addressed below in alphabetical order.

In transcriptome analyses, ABS has been found to increase the expression of *cyclic AMP response element-binding protein* (*CREB*)/ATF BZIP transcription factor (CREBZF), a member of the mammalian CREB family of transcription factors; this increased the level of the anti-neoplastic protein p53, thereby enhancing gene transcription.[45] CREBZF also regulates the unfolded protein response to protect against excessive protein synthesis during endoplasmic reticulum stress.[46]

Hepatocyte nuclear factor (HNF)-4a is a nuclear receptor found not only in the liver but also in other tissues, which is involved in embryonic development, cellular differentiation, and hepatocyte-specific protein synthesis. Recent studies have shown that HNF-4a also has anti-neoplastic activity, with its inhibition leading to tumor growth.[47] HNF-4a is a component of ABS extract and may be partly responsible for its anti-tumorigenic effects.

Malic enzyme (ME)-1 is an intracellular cytosolic protein and a component of ABS that converts malic acid to pyruvic acid, yielding nicotinamide adenine dinucleotide phosphate (NAPDH). ME-1 plays an important role in cancer metabolism, since NADPH is required for anaerobic respiration; indeed, ME-1 level is upregulated in some cancers.[48,49] However, the role of ME-1 in ABS remains unclear given that it remains outside of cells, and its in vivo function has not been investigated.

Midkine is a heparin-binding protein that is involved in cellular growth, survival, migration, and differentiation. Midkine was found to suppress vascular endothelial growth factor A, which plays a key role in tumoral angiogenesis; its inhibition may thus inhibit tumor growth.[50,51] Although a more likely role for midkine is the promotion of tissue healing (discussed below), we speculate that it also has anti-tumoral effects.

Protein inhibitor of activated signal transducer and activator of transcription (PIAS)-2 is another component of ABS extract. This protein belongs to PIAS family, whose members suppress the activity of STAT proteins,[52] which are critical components of the Janus kinase (JAK) cascade that acts downstream of many growth factor receptors. JAK-STAT signaling is a major pathway involved in human carcinogenesis.[53] Thus, the anti-neoplastic action of ABS may be exerted via inhibition of STAT protein via PIAS-2.

Table 2. Hypothesized mechanisms of individual components' contributions on Ankaferd's pleiotropic effects.

Pleiotropic effect	Component	Relevance
Anti-neoplastic effects	CREBZF	Increases p53 transcription
	PIAS2	Inhibits activity of STAT proteins
	HNF-4a	Its inhibition promotes tumor growth
	ME-1	It converts malic acid to pyruvic acid resulting in production of NADPH
	P18INK4C	Inhibits CDKs
	Midkine	Downregulates VEGF-A
Tissue-healing effects	Dynactin	Has a filamentous structure and potential to integrate with other structures in vivo
	Egr-1	Has an important role in cell proliferation and differentiation
	Midkine	Promotes endothelial cellular proliferation and angiogenesis
	C-myc	Plays a key role in progression of cell cycle, cellular growth, cellular transformation
	NF-1	Inhibits RAS and regulates growth and differentiation of keratinocytes and its increased expression is shown in healing tissues in human epidermis
	Twinfilin	Interacts with extracellular actin to promote protein scaffold for tissue healing
	YY1	Promotes cellular differentiation, proliferation, and growth, also is shown to protect from apoptosis
Antimicrobial effects	Tocotrienols vitamin E family	Have distinct antioxidant effects
	Galangin	The topoisomerase IV enzyme may therefore be implicated in the antibacterial mechanism of action of galangin
	Apigenin	Has an antioxidant, anticarcinogenic, and spasmolytic activities and can reduce high blood pressure
	Tertiary butylhydroquinone (TBHQ)	Prompts loss of staphylococcal membrane integrity
	BHT (butylated hydroxytoluene)	Has an antioxidant effect

The cyclin-dependent kinase (CDK) inhibitor P18INK4C[54] inhibits tumorigenesis, and its deficiency promotes tumor growth.[55,56] CDKs are serine/threonine kinases that regulate the cell cycle and thus play a vital role in human cancers. P18INK4C may participate in the tumor-suppressor activity of ABS by inhibiting CDKs.

Tissue-healing effects

In addition to being a hemostatic agent, ABS has an established role in promoting tissue repair after radiation damage and colitis;[57] bone, cartilage, muscle, and tendon remodeling;[27,58,59] and repair of dermal and epidermal tissue,[29] gastrointestinal mucosa, and full-thickness injuries in the abdominal viscera.[9,60] We hypothesize that Dynactin, Egr-1, Midkine, NF-1, Twinfilin, V-myc, and Yin Yang 1 can contribute to this effect thus mentioned in following paragraphs with possible mechanisms.

Dynactin along with its partner motor protein dynein is an integral component of cytoskeletal machinery that controls organelle movement during cell division. Dynactin has a central alpha helix with adjacent peptides that interact with dynein and other proteins. Dynactin is an ABS constituent; given its filamentous structure and capacity for integrating with other structures in vivo, it may contribute to the stimulatory effect of ABS on tissue healing.[61,62]

Inflammation is an integral aspect of tissue repair. Early growth factor (Egr)-1 is a transcription factor and ubiquitous growth factor present in ABS that has an important role in cell proliferation and differentiation. Increasing Egf-1 transcriptional activity may promote tissue regeneration.[63,64]

Midkine, the heparin-binding protein described above, is known to stimulate the growth of and provide protection to tissue,[65] but also regulates immunity and inflammation. Midkine promotes endothelial cell proliferation and angiogenesis and has been implicated in the pathogenesis of various diseases.[66]

Neurofibromin (NF)-1 is a tumor suppressor whose mutation is linked to neurofibromatosis type 1 and juvenile myelomonocytic leukemia. It is a Ras GTPase-activating protein that inhibits Ras and regulates the growth and differentiation of keratinocytes; it is overexpressed in the human epidermis during tissue repair.[67,68]

Twinfilin is an actin monomer-binding protein that stabilizes and facilitates the function of actin fibrils.[69] It is highly conserved between yeasts, humans, and other mammals. Twinfilin in ABS may interact with extracellular actin to form a protein scaffold that facilitates tissue repair.[70]

V-myc avian myelocytomatosis viral oncogene homolog (c-myc) and its product nuclear c-Myc phosphoprotein play a key role in cell cycle progression and cell growth and transformation. Although c-myc is a known oncogenic protein, its

transcription is upregulated during wound healing;[71] as such, it may be another ABS component that contributes to tissue repair.

Yin Yang 1 is a ubiquitous and highly conserved transcription factor enriched in ABS that is known to promote cell differentiation, proliferation, and growth, especially in the central nervous system, and protect against apoptosis.[72]

Conclusion

The aim of this review is to constitute a framework for future research about pleiotropic effects of ABS. It is composed of hypotheses of Ankaferd ingredients' individual contributions to pleiotropic effects. There are some limitations of this review. The hypotheses are constructed regarding the molecules' actions in literature. First, these effects may not be reproduced in vivo and within the environment that the drug acts. Discrete studies on each ingredient is needed to confirm isolated effects of these molecules. Second, there may be some alternative explanations for these effects including contribution of other molecules or divergent effects of known molecules. Third, the process for each pleiotropic action needs to be explained more precisely with more studies to enable better assumptions and a more straightforward research tract about underlying molecular processes.

Ankaferd is an effective hemostatic agent that has been shown to be effective in suppressing gastrointestinal, dental, urologic, oropharyngeal, thoracic, and dermal bleeding in clinical trials. Animal studies and case reports have demonstrated the pleiotropic effects of Ankaferd that may have therapeutic benefits, although there have been no controlled clinical trials evaluating this in humans. We hypothesized that specific factors in ABS contribute to this pleiotropism (Table 2). Additional studies investigating these individual components can broaden the therapeutic potential and applicability of ABS.

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