In Vitro Anti-Bacterial Activities of Ankaferd Medicinal Plant Extract

Ankaferd Tıbbi Bitki Ekstresinin İn Vitro Antibakteriyel Aktiviteleri

Nefise AKKOÇ,[a]
Mustafa AKÇELİK,[a]
İbrahim C. HAZNEDAROĞLU, MD,[b]
Hakan GÖKER, MD,[b]
Mehmet TURGUT, MD,[c]
Salih AKSU, MD,[b]
Şerafettin KİRAZLI,[d]
H. Cahit FIRAT[d]

[a]Department of Biology,
Ankara University Faculty of Science,
[b]Department of Hematology,
Hacettepe University Faculty of Medicine,
Ankara
[c]Department of Hematology,
Ondokuz Mayıs University
Faculty of Medicine, Samsun
[d]Department of
Research&Development,
Ankaferd Drug Inc., İstanbul

Geliş Tarihi/*Received:* 19.05.2008
Kabul Tarihi/*Accepted:* 16.12.2008

Congress Abstract form of this article was presented in the Meeting Supplement of Int J Lab Hematol (2008;30:95).

Yazışma Adresi/*Correspondence:*
İbrahim C. HAZNEDAROĞLU, MD
Hacettepe University
Faculty of Medicine,
Department of Hematology, Ankara,
TÜRKİYE/TURKEY
haznedar@yahoo.com

ABSTRACT Objective: The medicinal value of plants lies in some chemical substrates that produce a definitive physiological action on the human body. Ankaferd comprises a standardized mixture of the plants *Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum* and *Urtica dioica*. The aim of this study was to assess the in vitro antimicrobial activities of Ankaferd medicinal plant extract. **Material and Methods:** The antimicrobial activity assay was performed by agar well diffusion to assess the antagonistic activity of Ankaferd against 26 indicator strains including human pathogens and food spoiling gram-negative and gram-positive bacteria. **Results:** Ankaferd was active against all bacteria tested while nisin, the only commercial bacteriocin for food preservation, was inactive against gram-negative indicator strains. Besides a high inhibitory activity against gram-positive and gram-negative bacteria, including human pathogens and food spoiling bacteria, Ankaferd was more stable than nisin in different heat and enzyme treatments. **Conclusion:** Antibacterial activity of Ankaferd can be extended to extreme environmental conditions such as the potential use of the preparation for the treatment of infectious diseases and preservation of different type foods from food-born pathogens or food spoiling bacteria. The demonstration of the anti-infective properties of Ankaferd adds a new value to its haemostatic effect in the healing of infected hemorrhagic wounds, as well as opening new avenues for its potential use for anti-infective actions and food preservation.

Key Words: Hemostasis; herb-drug interactions; gram-negative bacteria; gram-positive bacteria

ÖZET Amaç: Bitkilerin tıp alanındaki önemleri bazı kimyasal substratların insan vücudunda yarattığı önemli fizyolojik etkilere dayanmaktadır. Ankaferd; *Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum* ve *Urtica dioica* bitkilerinin belirli ölçülerde karışımlarını ihtiva etmektedir. Çalışmanın amacı Ankaferd tıbbi bitki ekstresinin in vitro antimikrobiyel aktivitesinin belirlenmesidir. **Gereç ve Yöntemler:** Antimikrobiyal aktivite araştırmaları; arasında insan patojenleri ve gıda bozulma etmeni gram-negatif ve gram-pozitif bakterilerin de bulunduğu 26 endikatör kökene karşı agar kuyu diffüzyonu yöntemi kullanılarak gerçekleştirilmiştir. **Bulgular:** Ticari olarak kullanımına izin verilen tek bakteriyosin olan nisin gram-negatif endikatör kökenlere karşı etkili olmaz iken, Ankaferd'in test edilen tüm bakterilere karşı etkinlik gösterdiği belirlenmiştir. Aralarında insan patojeni ve gıda bozulma etmeni bakterilerin de bulunduğu gram-pozitif ve gram-negatif bakterilere karşı yüksek inhibisyon aktivitesi göstermesinin yanı sıra, Ankaferd farklı sıcaklık ve enzim muameleleri sonucunda nisinden daha dayanıklı bulunmuştur. **Sonuç:** Ankaferd'in antibakteriyel aktivitesinden, enfeksiyon hastalıklarının tedavisinde preparat olarak ve gıda kaynaklı patojenlerden ya da gıda bozulmasına neden olan bakterilerden gıdaların korunması gibi pek çok geniş çevresel koşullarda yararlanılabilir. Ankaferd'in anti-enfektif özelliklerinin belirlenmesi, enfekte olmuş hemorajik yaralara hemostatik etkilerine yeni değerler kazandıracağı gibi, gıda korunmasında ve anti-enfektif etkilerinde de yeni açılımlar oluşturacaktır.

Anahtar Kelimeler: Hemostaz; ilaç-bitki etkileşimi; gram-negatif bakteriler; gram-pozitif bakteriler

Turkiye Klinikleri J Med Sci 2009;29(2):410-5

The use of plant extracts and phytochemicals, with established antimicrobial properties, could be of great significance in preventive and/or therapeutic approaches. The most important antimicrobial compounds of plants are alkoloids, flavonoids, tannins and phenolic compounds.[1-4] The increasing prevalence of multi-drug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raised the specter of "untreatable" bacterial infections and adds urgency to the search for new infection-fighting strategies.[5,6] In contrast to synthetic drugs, antimicrobials of plant origin usually are not associated with many side effects and have an enormous anti-infective potential in numerous infectious diseases.[2,7,8]

Ankaferd is a unique folkloric medicinal plant extract, which has historically been used in Turkish traditional medicine as a haemostatic agent. Ankaferd comprises a standardized mixture of the plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum* and *Urtica dioica*. The basic mechanism of action of the pharmacological preparation of Ankaferd Blood Stopper® is the formation of an encapsulated protein network that provides focal points for erythrocyte aggregation. Exposure to Ankaferd seems to provide a tissue oxygenation as well as a physiological haemostatic process without affecting any individual clotting factor.[9] The aim of this study was to assess *in vitro* the antimicrobial activities of Ankaferd medicinal plant extract. Elucidation of the anti-infective properties of Ankaferd adds a new value to its haemostatic effect in the healing of infected hemorrhagic wounds, as well as opening new avenues for its potential use for anti-infective actions and food preservation.

MATERIAL AND METHODS

BACTERIAL STRAINS

The bacteria used in this study were propagated in appropriate media as indicated in Table 1. Bacterial stocks were stored at -80°C in their respective broths supplemented with 30% glycerol.

ANTIMICROBIAL ACTIVITY ASSAY

The antimicrobial activity assay was performed by agar well diffusion method.[10] Nutrient agar was inoculated with the given microorganisms by spreading the bacterial inoculums on the media. Wells were prepared in the plates with the help of a cork-borer (0.85 cm) and 100 µL of the test compound was introduced into the well. Standard nisin solution (Sigma, Deinsenhofen, Germany) (control) was also run parallel in the same plate. The plates were incubated overnight at appropriate incubation temperatures for indicator strains. Microbial growth was determined by measuring the diameter of zone of inhibition.

THE EFFECT OF HEAT AND ENZYMES ON MEDICINAL PLANT EXTRACT, ANKAFERD

The samples of Ankaferd (one vial of 100 mL) were provided by Ankaferd *Drug Inc.,* (Ankaferd patent number 2007-0-114485; Trend Teknoloji İlaç AS, Istanbul, Turkey, *www.*Ankaferd.*com*). To determine the effect of heat on Ankaferd, test samples were heated at 80°C, 90°C, 100°C and 121°C for 15 minutes. Ankaferd was also treated with the following enzymes at a final concentration of 1 $mg.mL^{-1}$: trypsin (pH 7.0, Sigma, Deinsenhofen, Germany), α-chymotrypsin (pH 7.0, Sigma), proteinase K (pH 7.0, Sigma), pepsin (pH 30, Merck, Darmstardt, Germany), α-amylase (pH 7.0, Sigma), lipase (pH 7.0, Sigma), catalase (pH 7.0, Sigma) and lysozyme (pH 7.0, Sigma). Following incubation at 37°C for 2 h, enzyme activity was terminated by heating at 100°C for 5 min. Untreated samples were used as controls. The inhibitory activities of treated and untreated samples were determined by the critical dilution method, using *Micrococcus luteus* NCIMB8166 as indicator.[11]

RESULTS AND DISCUSSION

ANTAGONISTIC ACTIVITY

Well diffusion assay was performed to assess the antagonistic activity of Ankaferd against 26 indicator strains including human pathogens and food spoiling, gram-negative and gram-positive bacteria. The folkloric medicinal extract, Ankaferd was active against all bacteria tested while nisin, the

TABLE 1: Inhibitory spectrum of *Ankaferd* medicinal plant extract.

Indicator strain	Inhibitory activity*		Source**	Media***
	Ankaferd	Nisin (1000 IU/mL)		
Lactococcus lactis subsp. *lactis* SIK-83 (nisin producer)	++	-	NHL	M17 (30°C 18 h)
Lactococcus lactis subsp. *lactis* ATCC7962 (nisin producer)	++	-	NHL	M17 (30°C 18 h)
Lactococcus lactis subsp. *lactis* LMG2908 (nisin producer)	++	-	NHL	M17 (30°C 18 h)
Micrococcus luteus NCIMB8166	+++	+++	AUFF	NB (30°C 18 h)
Bifidobacter bifidum CHL17	+++	++	NHL	MRS (37°C 18 h)
Bifidobacter longum CHL21	+++	++	NHL	MRS (37°C 18 h)
Lactobacillus sake NCDO2714	++	++	NHL	MRS (30°C 18 h)
Lactobacillus plantarum LMG2003	++	++	NHL	MRS (37°C 18 h)
Leuconostoc carnosum DSM5576	++	++	NHL	MRS (30°C 18 h)
Enterococcus faecium	++	++	AUFF	MRS (37°C 18 h)
Enterococcus faecalis LMG2602	++	++	NHL	MRS (37°C 18 h)
Staphylococcus aureus ATCC6538	+++	++	NHL	NB (37°C 18 h)
Staphylococcus carnosus MC1B	+++	++	NHL	NB (37°C 18 h)
Clostridium tyrobutyricum	+++	++	NHL	RCM (30°C 18 h)
Clostridium sporogenes	+++	++	NHL	RCM (30°C 18 h)
Bacillus subtilis 12	++	+++	NHL	NB (37°C 18 h)
Bacillus licheniformis 40	++	+++	NHL	NB (37°C 18 h)
Bacillus cereus LMG2732	++	+++	NHL	NB (30°C 18 h)
Pseudomonas fluorescens P1	+++	-	NHL	NB (37°C 18 h)
Pseudomonas aeruginosa ATCC15442	+++	-	NHL	NB (37°C 18 h)
Escherichia coli CFA1	+++	-	NHL	LB (37°C 18 h)
Salmonella enterica typhimurium	+++	-	NHL	LB (37°C 18 h)
Klebsiella pneumoniae	++	-	AUFF	NB (37°C 18 h)
Pediococcus pentosaceus LMG2001	++	++	NHL	MRS (37°C 18 h)
Listeria innocua 2813	++	+++	NHL	NB (30°C 18 h)
Listeria monocytogenes ATCC15313	++	+++	NHL	NB (30°C 18 h)

*- , no inhibition zone; ++, 5 mm ≤ inhibition zone ≤ 10 mm, +++, > 10 mm inhibition zone,

** NHL, Agricultural University of Norway, As, Norway; AUFF; Faculty of Science, University of Ankara, Ankara, Turkey,

*** MRS: de Man-Rogosa-Sharpe; LB: Luria-Bertani; NB: Nutrient broth, RCM: Reinforced clostridial medium (Merck, Damstardt, Germany). All bacteria were grown aerobically except obligate anaerobic *Clostridium strains.*

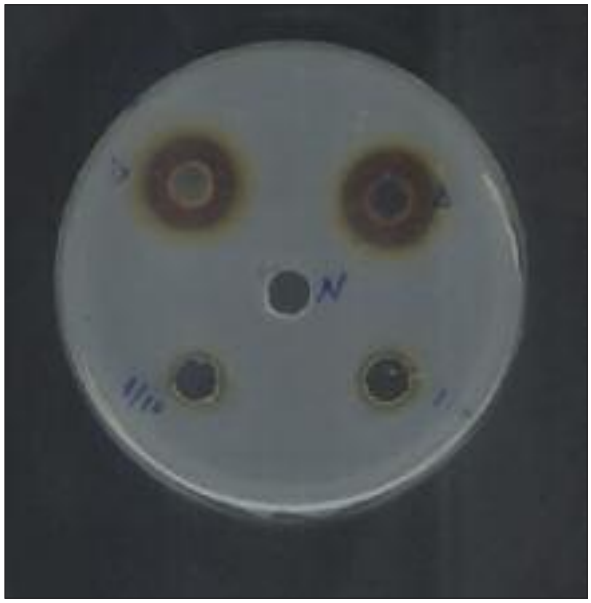

FIGURE 1: Inhibition zones of *Ankaferd* against *Salmonella enterica Typhimurium*. [D: Non diluted Ankaferd plant extract (+++), N: Nisin (control), 1/10: Dilution rate of Ankaferd plant extract (+)].

TABLE 2: Effect of enzymes and heat treatment on the Ankaferd medicinal plant extract.

Treatment	Inhibitory activity*	
	Ankaferd	Nisin (1000 IU/mL)
Enzymes		
Trypsin (Sigma, No. T8658)	+++	+++
α-chymotrypsin (Sigma, No. C-6423)	+++	-
Proteinaz K (Sigma, No. P-6390)	+++	-
Pepsin (Merck, No. 7147)	+++	+++
α-amylase (Sigma, typ VII A)	+++	+++
Lipase (Sigma, No. L17714)	+++	+++
Catalase (Sigma, No. C-10)	+++	+++
Lysozyme (Sigma, No. L7651)	+++	+++
Heat		
15 min at 80°C	+++	+++
15 min at 90°C	+++	+++
15 min at 100°C	+++	++
15 min at 121°C	+++	+

* Inhibitory activity was determined by using the indicator strain *Micrococcus luteus* NCIMB8166.

only commercial bacteriocin for food preservation, was inactive against gram-negative indicator strains (Table 1, Figure 1).

Gram-positive bacterial strains were more susceptible to the medicinal plant extracts as compared to gram-negative bacteria.[12,13] Ankaferd showed a high inhibitory activity for both gram-negative and gram-positive bacteria. This unique property of the Ankaferd medicinal plant extract pointed out its antimicrobial potential as a drug and foods additive.

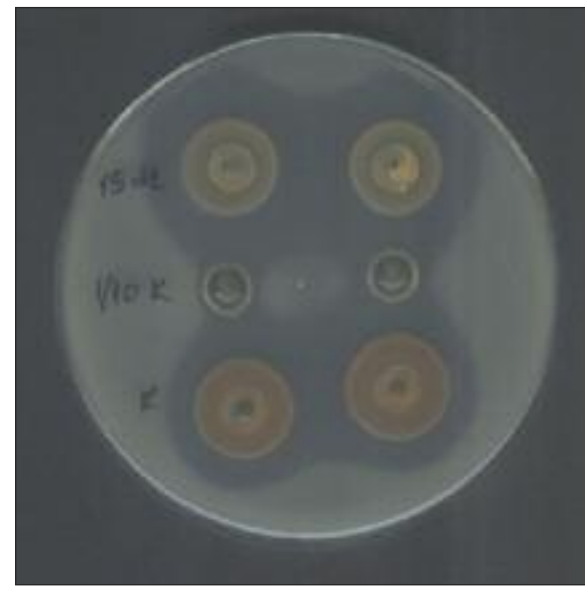

FIGURE 2: Inhibition zones of Ankaferd against *Micrococcus luteus* NCIMB8166 after treatment of 15 min at 121°C.
[15 min: Heat treatment time of *Ankaferd* plant extract (15 min, +++), 1/10: Dilution rate of *Ankaferd* plant extract (+), K: Heat untreated *Ankaferd* plant extract (control, +++)].

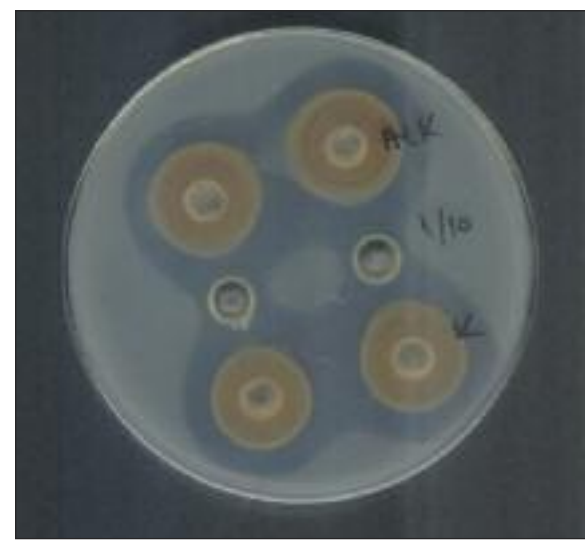

FIGURE 3: Inhibition zones of Ankaferd against *Micrococcus luteus* NCIMB8166 after proteinase K treatment.
[PrtK: Proteinase K treated *Ankaferd* plant extract (+++), 1/10: Dilution rate of *Ankaferd* plant extract (+), K: Proteinase K untreated *Ankaferd* plant extract (control, +++)].

EFFECT OF HEAT AND ENZYMES ON ANKAFERD

The enzymatic activity and heat stability of the Ankaferd compared to that of nisin was used an experimental control. The Ankaferd medicinal extract, was heat stable and was not affected by any enzymes tested whereas control bacteriocin nisin activity was completely lost after α-chymotrypsin and proteinase K treatment (Table 2, Figure 2, 3).

In addition to a broad-spectrum activity against gram-positive and gram-negative bacteria, including human pathogens and food spoiling bacteria, Ankaferd was more stable than nisin against various heat and enzyme treatments. The results presented here showed that the antibacterial activity of Ankaferd could be adapted to extreme environmental conditions such as potential use of the preparation for treatment of infectious diseases and preservation of different types of food from food-born pathogens or food spoiling bacteria. When added to plasma or serum, Ankaferd induces very rapid formation of a protein network and erythrocyte aggregation.[9] Sequelae of severe infection and sepsis may cause accelerated clearance of erythrocytes from circulating blood and the exposure of erythrocytes to pathogens can induce eryptosis.[14] Furthermore, alterations in red blood cell function can contribute to pathological changes in microcirculatory blood flow and cellular dysoxia in sepsis.[15] In a recent study, the haemostatic mechanism-of-action of the Ankaferd Blood Stopper®, was investigated.[9] Ankaferd stimulated the formation of an encapsulated protein network that provided focal points for erythrocyte aggregation.[9] Since infection and bleeding coexist in a variety of disorders,[16-18] Ankaferd has the therapeutic potential to be used for the management of haemorrhage in these difficult clinical conditions. Haemostatic process and bacterial infections have significant cross talks regarding biological basis[19-23] and clinical grounds.[24-26] Our present results indicated that future studies on Ankaferd should focus on not only the haemostatic actions but also on anti-infective properties of this unique medicinal plant extract in health and disease. There are preliminary evidences supporting this hypotheses such as successful endoscopic application of Ankaferd in upper gastrointestinal bleeding,[27] and mediastinal bleedings after coronary artery bypass graft operations in cardiac surgery.[28] Ankaferd Blood Stopper provides a therapeutic potential for the management of patients with deficient primary hemostasis in clinical medicine with its in vivo hemostatic actions. Ankaferd promotes the very rapid (< 1 second) formation of a specific protein network which acts as an anchor for vital erythrocyte aggregation, covering the classical clotting cascade.[9] Phase I studies are completed and Ankaferd is currently being studied in the treatment of Kırım-Kongo hemorrhagic fever with promising preliminary results, based on its anti-infective and hemostatic efficacy[28-30] even with defective platelets and/or coagulation factors. Very recently, Taşdelen-Fışgın et al assessed the *in vitro* antimicrobial activity of Ankaferd on 102 clinical isolates of gram-negative and gram-positive bacteria and four standard strains, including MRSA ATCC 43300, MSSA ATCC 25923, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 35218. ABS was significantly active against all bacteria investigated in their study.[31] The effects of Ankaferd protein library on vascular endothelium,[32] blood cells, angiogenesis, cellular proliferation, vascular dynamics and cellular mediators are currently being investigated to determine its potential role in many pathological states, including neoplastic disorders, infectious diseases, inflammation, premature aging, and atherosclerosis.

CONFLICT OF INTEREST

Ankaferd is a traditional folkloric medicinal plant extract that has been developed by HC Fırat.

REFERENCES

1. Ateş DA, Erdoğrul Ö. Antimicrobial activities of various medicinal and commercial plant extracts. Turk J Biol 2003;27(3):157-62.
2. Duraipandiyan V, Ayyanar M, Ignacimuthu S. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. BMC Complement Altern Med 2006;6: 35.
3. Mahasneh AM, El-Oqlah AA. Antimicrobial activity of extracts of herbal plants used in the traditional medicine of Jordan. J Ethnopharmacol 1999;64(3):271-6.
4. Nascimento GG, Locatelli J, Freitas PC, Silva GL. Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Brasilian J Microbiol 2000;31(4): 247-56.
5. Rojas JJ, Ochoa VJ, Ocampo SA, Muñoz JF. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in the treatment of non-nosocomial infections. BMC Complement Altern Med 2006;6:2.
6. Zy EA, Area A, Aam K. Antimicrobial activity of some medicinal plant extracts in palestine. Pak J Med Sci 2005;21(2):187-93.
7. Duraipandiyan V, Ignacimuthu S. Antibacterial and antifungal activity of Cassia fistula L: an ethnomedicinal plant. J Ethnopharmacol 2007;112(3):590-4.
8. Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. J Appl Microbiol 1999;86(6):985-90.
9. Goker H, Heznedaroglu C, Ercetin S, Kirazli S, Akman U, Oztürk Y, et al. Haemostatic actions of the folkloric medicinal plant extract Ankaferd Blood Stopper. J Int Med Res 2008; 36(1):163-70.
10. Perez C, Paul M, Bazerque P. An antibiotic assay by the agar well diffusion method. Acta Bio Med Exp 1990;15:113-5.
11. Franz CM, Du Toit M, von Holy A, Schillinger U, Holzapfel WH. Production of nisin-like bacteriocins by Lactococcus lactis strains isolated from vegetables. J Basic Microbiol 1997;37(3): 187-96.
12. Parekh J, Chanda S. In-vitro antimicrobial activities of extracts of launaea procumbens roxb. (labiateae), vitis vinifera l. (vitaceae) and cyperus rotundus l. (cyperaceae). Afr J Biomed Res 2006;9:89-93.
13. Rabe T, van Staden J. Antibacterial activity of South African plants used for medicinal purposes. J Ethnopharmacol 1997;56(1):81-7.
14. Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, et al. Suicidal erythrocyte death in sepsis. J Mol Med 2007;85(3): 273-81.
15. Piagnerelli M, Boudjeltia KZ, Vanhaeverbeek M, Vincent JL. Red blood cell rheology in sepsis. Intensive Care Med 2003;29(7):1052-61.
16. Büyükaşik Y, Ileri NS, Haznedaroğlu IC, Demiroğlu H, Dündar S. Fever, hepatosplenomegaly and pancytopenia in a patient living in the Mediterranean region. Postgrad Med J 1998;74(870):237-9.
17. Büyükaşik Y, Ozcebe OI, Sayinalp N, Haznedaroğlu IC, Altundağ OO, Ozdemir O, et al. Perianal infections in patients with leukemia: importance of the course of neutrophil count. Dis Colon Rectum 1998;41(1):81-5.
18. Gonen C, Uner A, Cetinkaya Y, Hascelik G, Haznedaroglu I. Tonsillar abscess formation due to herpes simplex type-1 in a severely immunocompromised stem cell transplant patient with chronic myeloid leukemia. Transpl Infect Dis 2006;8(3):166-70.
19. Bergmann S, Hammerschmidt S. Fibrinolysis and host response in bacterial infections. Thromb Haemost 2007;98(3):512-20.
20. Choi G, Vlaar AP, Schouten M, Van't Veer C, van der Poll T, Levi M, et al. Natural anticoagulants limit lipopolysaccharide-induced pulmonary coagulation but not inflammation. Eur Respir J 2007;30(3):423-8.
21. Feistritzer C, Wiedermann CJ. Effects of anticoagulant strategies on activation of inflammation and coagulation. Expert Opin Biol Ther 2007;7(6):855-70.
22. Gonçalves S, Santos NC, Martins-Silva J, Saldanha C. Fibrinogen-beta-estradiol binding studied by fluorescence spectroscopy: denaturation and pH effects. J Fluoresc 2006;16(2): 207-13.
23. Matejovic M, Krouzecky A, Radej J, Rokyta R Jr, Kralova H, Radermacher P, et al. Coagulation and endothelial dysfunction during long-term hyperdynamic porcine bacteremia-- effects of selective inducible nitric oxide synthase inhibition. Thromb Haemost 2007;97 (2): 304-9.
24. Cornet AD, Smit EG, Beishuizen A, Groeneveld AB. The role of heparin and allied compounds in the treatment of sepsis. Thromb Haemost 2007;98(3):579-86.
25. Gonen C, Haznedaroğlu IC, Aksu S, Koca E, Göker H, Büyükaşik Y, et al. Endogenous thrombopoietin levels during the clinical management of acute myeloid leukaemia. Platelets 2005;16(1):31-7.
26. Lissalde-Lavigne G, Combescure C, Dorangeon E, Lefrant JY, Gris JC. Simple coagulation tests improve early mortality prediction for patients in intensive care units who have proven or suspected septic shock. J Thromb Haemost 2007;5(5):1081-3.
27. Kurt M, Disibeyaz S, Akdogan M, Sasmaz N, Aksu S, Haznedaroglu IC. Endoscopic application of ankaferd blood stopper as a novel experimental treatment modality for upper gastrointestinal bleeding: a case report. Am J Gastroenterol 2008;103(8):2156-8.
28. Dogan OF, Ozyurda U, Uymaz OK, Ercetin S, Haznedaroglu IC. New anticoagulant agent for CABG surgery. Eur J Clin Invest 2008;38(5): 341.
29. Kurt M, Kacar S, Onal IK, Akdogan M, Haznedaroglu IC. Ankaferd Blood Stopper as an effective adjunctive hemostatic agent for the management of life-threatening arterial bleeding of the digestive tract. Endoscopy 2008;40(Suppl 2):E262.
30. Ibis M, Kurt M, Onal IK, Haznedaroglu IC. Successful management of bleeding due to solitary rectal ulcer via topical application of Ankaferd blood stopper. J Altern Complement Med 2008;14(9):1073-4.
31. Tasdelen Fisgin N, Tanriverdi Cayci Y, Coban AY, Ozatli D, Tanyel E, Durupinar B, et al. Antimicrobial activity of plant extract Ankaferd Blood Stopper. Fitoterapia 2009;80(1): 48-50.
32. Haznedaroğlu İC. [Mechanisms of hemostasis]. Turkiye Klinikleri J Int Med Sci 2005;1(2): 1-5.