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Original Article

Evaluation of the in vivo Antifungal Effects of Ankaferd Blood Stopper in a Neutropenic Rat Model of Systemic Candidiasis

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ABSTRACT

Background This study aimed to evaluate the in vivo antifungal effects of Ankaferd Blood Stopper (ABS) in a neutropenic rat model of systemic *Candida albicans* infection.

Methods A total of 24 Wistar-Albino rats were divided into two groups: one receiving ABS and the other receiving normal saline (control group). Neutropenia was induced using cyclophosphamide, followed by intraperitoneal C. albicans inoculation. The ABS group received 1 mL of ABS via an orogastric tube, while the control group received 1 mL of saline. The rats were monitored for clinical changes, and histopathological evaluations were conducted on liver, lung, and kidney tissues.

Results While no significant antifungal effect was observed in the lung (p=0.590) and kidney tissues (p=1.000), ABS-treated rats showed statistically significant lymphoid infiltration (p=0.014) and hepatocyte regeneration in liver tissue (p=0.001). This suggests that ABS may enhance immune response and promote tissue healing.

Conclusion In this regard, our study is the first and only study to evaluate the in vivo antifungal efficacy of Ankaferd. The findings indicate that ABS may have immunomodulatory effects in systemic candidiasis, particularly in liver tissue. However, further studies are required to elucidate its pharmacokinetics and optimal dosing for antifungal efficacy.

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INTRODUCTION

Ankaferd Blood Stopper (ABS) is a plant extract used as a hemostatic agent in Turkish medicine.[1] It is a standardized mixture of Thymus vulgaris (thyme), Glycyrrhiza glabra (licorice root), Vitis vinifera (grapevine), Alpinia officinarum (galangal), and Urtica dioica (stinging nettle).[1, 2] ABS facilitates hemostasis by rapidly forming an encapsulated protein network without affecting physiological coagulation mechanisms, leading to erythrocyte aggregation within seconds. It is the first Turkish product licensed by the Ministry of Health for topical use in controlling external bleeding and hemorrhage in dental surgery.[3, 4]. In addition to its hemostatic properties, various in vivo studies have demonstrated ABS's antibacterial, antifungal, and antiviral activities against numerous pathogens, yielding positive results.[5-8] *Candida albicans* and other Candida species cause opportunistic infections, particularly in immunocompromised patients, contributing to significant morbidity and mortality.[9] The emergence of resistance to antifungal drugs and the limited efficacy of current treatment options necessitate the search for new antifungal agents. Studies on the potential antifungal effects of plant-derived preparations play a crucial role in shaping future treatment strategies. In this context, since the potential antifungal properties of ABS may be beneficial, we investigated its pathological effects on liver, lung, and kidney tissues in a neutropenic rat model of systemic *C. albicans* infection.

MATERIAL AND METHODS

This experimental study was approved by the Ethics Committee for Animal Research of İnönü University Faculty of Medicine, and all procedures were applied in accordance with the principles of the National Guidelines for Experimental Use of Laboratory Animals (Protocol No: 2013/101).

Animals

The study was conducted at the İnönü University Experimental Animal Production and Research Laboratory using 24 Wistar-Albino rats of both sexes, weighing approximately 180–200 g. The rats were housed under standard laboratory conditions with rat chow and fresh tap water. The temperature was maintained at 22 ± 2°C, and humidity at 45%. The rats were divided into two groups and placed in separate cages in groups of four. The rats were assigned to two groups: one receiving ABS and the other receiving physiological saline (control group) .

Immunosuppression and Supportive Care

To induce neutropenia, intraperitoneal cyclophosphamide injections were administered at a dose of 90 mg/kg body weight five days before C. albicans inoculation and 60 mg/kg body weight one day before inoculation. Neutropenia was confirmed in all rats by the İnönü University Hematology Laboratory. To maintain neutropenia, an additional intraperitoneal injection of cyclophosphamide at a dose of 60 mg/kg body weight was administered to all rats three days after C. albicans inoculation (10).

To prevent secondary bacterial infections, all rats received intramuscular amoxicillin at a dose of 40 mg/kg for seven days starting on day five of the study. Additionally, on day six following C. albicans inoculation, intramuscular gentamicin was administered at a dose of 40 mg/kg. Furthermore, ciprofloxacin and polymyxin E were added to the drinking water of all rats throughout the study period (10).

Experimental Systemic Candidiasis

To induce systemic Candida infection, the C. albicans (ATCC 10231) strain was used. On the sixth day of the study, the C. albicans (ATCC 10231) strain was subcultured on Sabouraud dextrose agar (SDA) and diluted in normal saline to a 0.5 McFarland standard. A 1-mL aliquot of the prepared C. albicans suspension was intraperitoneally injected into each of the neutropenic rats. Following C. albicans inoculation, 12 rats designated to receive ABS were administered 1 mL of ABS via an orogastric tube, while 12 control group rats received 1 mL of normal saline via syringe (10). The rats were returned to their cages and monitored for one week. Health status was assessed twice daily based on behavioral changes, activity levels, posture control, mobility, and daily food and water intake.

Tissue Procurement and Histopathological Examination

On day 13, all rats underwent general anesthesia via a single subcutaneous injection of ketamine HCl (40 mg/kg) and xylazine HCl (15 mg/kg). Once anesthesia was achieved, the rats were positioned in a prone position on the operating table, and their

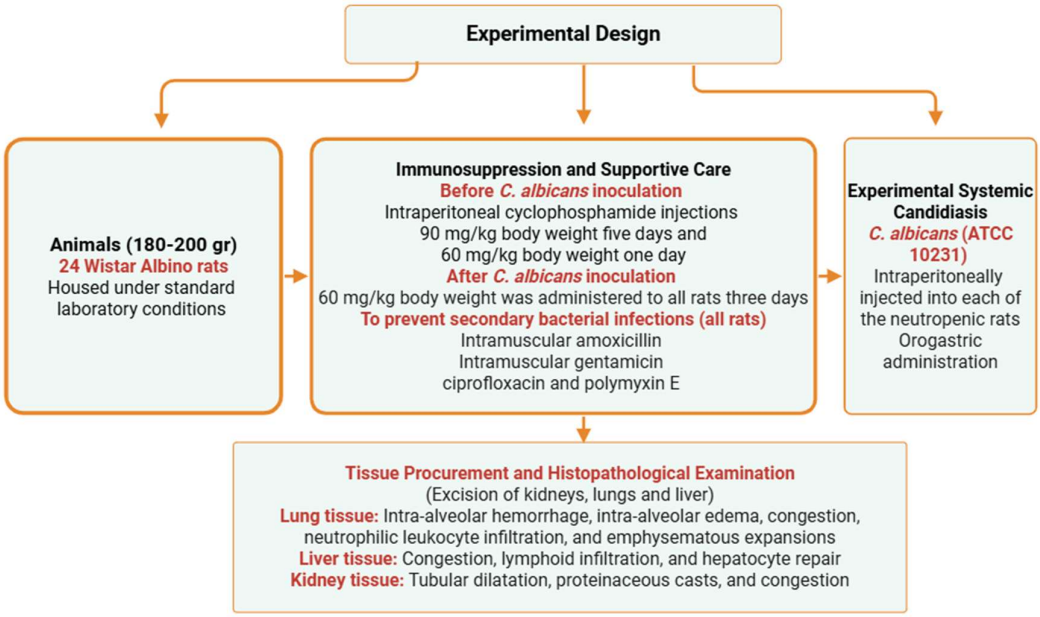

Figure 1. Design of the Study

kidneys, lungs, and liver were excised. The rats were then sacrificed. The excised organs were placed in numbered containers filled with 10% formaldehyde and sent to the Department of Pathology for histopathological examination. Tissue samples were embedded in paraffin blocks, sectioned at a thickness of 5 µm, and stained with hematoxylin-eosin. Sections were examined under a light microscope at 40× magnification. Two pathologists, blinded to the study groups, evaluated the specimens. Histopathological findings were graded as follows: 0 (normal) , 1 (mild to moderate) , and 2 (severe) .

The histopathological evaluation included:

- Lung tissue: Intra-alveolar hemorrhage, intra-alveolar edema, congestion, neutrophilic leukocyte infiltration, and emphysematous expansions.
- Liver tissue: Congestion, lymphoid infiltration, and hepatocyte repair.
- Kidney tissue: Tubular dilatation, proteinaceous casts, and congestion.

Figure 1 represents the methodological design of the study

Statistical analysis

Statistical analysis was performed using SPSS 15.0. Continuous variables were presented as mean ± standard deviation (SD) . Categorical variables were compared between groups using chi-square analysis. A p-value of <0.05 was considered statistically significant.

RESULTS

Rats were divided into two groups based on the administered substance: Group 1 (rats receiving saline solution) and Group 2 (rats receiving ABS). Findings were classified into three categories: 0 (normal), 1 (mild to moderate), and 2 (severe).

Findings in Liver Tissue

A. Congestion: Mild to moderate congestion was observed in the liver tissue of all 12 rats in the saline group and in 11 out of 12 rats in the ABS group. However, severe congestion was not detected in either group. There was no statistically significant difference in liver congestion between the groups (p = 1.000).

B. Lymphoid Infiltration: Lymphoid infiltration in the liver parenchyma was detected at a mild to moderate level in all 12 rats of the saline group. In contrast, 6 out of 12 rats in the ABS group exhibited mild to moderate lymphoid infiltration, while the remaining 6 showed severe infiltration. A statistically significant difference in lymphoid infiltration was found between the groups (p = 0.014).

C. Hepatocyte Repair: Hepatocyte repair was observed in only 1 of the 12 rats in the saline group, whereas no repair was detected in the remaining 11 rats. In the ABS group, hepatocyte repair was observed in 11 out of 12 rats, with only one rat showing no signs of repair. A statistically significant difference in

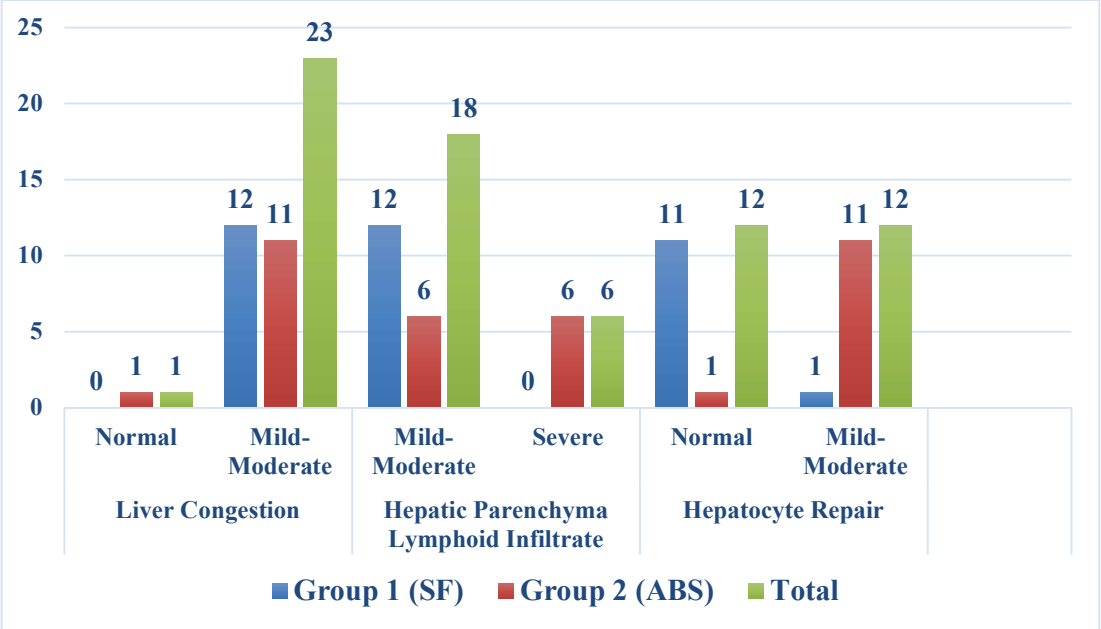

Figure 2. Comparison of Histopathological Changes in Liver Tissue of Rats Given ABS and SF

hepatocyte repair was found between the groups (p = 0.001). The histopathological findings in liver tissue are shown in Figure 2.

Findings in Lung Tissue:

A. Intraalveolar Hemorrhage: In the saline group, intraalveolar hemorrhage was observed in 3 out of 12 rats, while in the Ankaferd group, a similar finding was noted in 1 out of 12 rats. No statistically significant difference was found between the groups regarding intraalveolar hemorrhage (p=0.590).

B. Intraalveolar Edema: In the saline group, intraalveolar edema was detected in 2 out of 12 rats, whereas in the Ankaferd group, intraalveolar edema was observed in 4 out of 12 rats. No statistically significant difference was found between the groups regarding intraalveolar edema (p=0.640).

C. Neutrophil Leukocyte Infiltration: In the saline group, mild neutrophil leukocyte infiltration was observed in 3 out of 12 rats, while a similar finding was noted in 6 out of 12 rats in the Ankaferd group. No statistically significant difference was found between the groups regarding neutrophil leukocyte infiltration (p=0.400).

D. Congestion: In the saline group, no congestion was observed in the lung tissue of 2 out of 12 rats, while congestion was seen in all rats in the Ankaferd group. No statistically significant difference was found between the groups regarding congestion (p=0.478).

E. Emphysematous Expansion: In the saline group, emphysematous expansion was observed in 2 out of 12 rats, while this finding was noted in all rats of the Ankaferd group. No statistically significant difference was found between the groups regarding emphysematous expansion (p=0.478).

The histopathological findings in lung tissue are shown in Table 1

Findings in Kidney Tissue:

Table 1. Comparison of Histopathological Changes in Lung Tissue of Rats Given ABS and SF

Groups	Intraalveolar Hemorrhage in the Lung		Intraalveolar Edema in the Lung		Neutrophil Leukocyte Infiltration in the Lung		Congestion in the Lung		Emphysematous Expansion in the Lung	
	0*	1*	0*	1*	0*	1*	0*	1*	0*	1*
Group 1 (SF)	9	3	10	2	9	3	2	10	2	10
Group 2 (ABS)	11	1	8	4	6	6	0	12	0	12
Total	20	4	18	6	15	9	2	22	2	22

*0: Normal, 1: Mild-Moderate, 2: Severe

Table 2. Comparison of Histopathological Changes in Kidney Tissue of Rats Given ABS and SF

	Tubular Dilation in the Kidney		Proteinaceous Casts in the Kidney		Congestion in the Kidney	
	0*	1*	0*	1*	0*	1*
Group 1 (SF)	6	6	1	11	6	6
Group 2 (ABS)	7	5	2	10	4	8
Total	13	11	3	21	10	14

* 0: Normal, 1: Mild-Moderate, 2: Severe

A. Tubular Dilation: In the saline group, tubular dilation was observed in 6 out of 12 rats, while in the Ankaferd group, tubular dilation was detected in 5 out of 12 rats. No statistically significant difference was found between the groups regarding tubular dilation (p=1.000).

B. Proteinaceous Casts: In the saline group, proteinaceous casts were observed in 11 out of 12 rats, while a similar finding was detected in 10 out of 12 rats in the Ankaferd group. No statistically significant difference was found between the groups regarding proteinaceous casts (p=1.000).

C. Congestion: In the saline group, renal congestion was observed in 6 out of 12 rats, while congestion was seen in 8 out of 12 rats in the Ankaferd group. No statistically significant difference was found between the groups regarding congestion (p=0.680). The histopathological findings in the kidney tissue are shown in Table 2

Considering these findings, although some differences were observed in the histopathological changes between the two groups, no statistically significant differences were found for most parameters.

DISCUSSION

Infections in neutropenic patients are an important caA review of the literature reveals no prior studies on the in vivo antifungal activity of ABS. In this regard, our study is the first and only study to evaluate the in vivo antifungal efficacy of Ankaferd. In addition to in vivo and in vitro studies on the hemostatic effects of Ankaferd, its antimicrobial activity has been investigated against various pathogens.[11] Beyond its antifungal properties, Ankaferd Blood Stopper (ABS) has also been investigated for its antimicrobial activity using various bacterial isolates, including *Streptococcus mutans*, *Staphylococcus aureus*, *Actinomyces israelii*, and *Lactobacillus casei.*[12]

Similarly, in another study, the in vitro antibacterial efficacy of Ankaferd against multidrug-resistant microorganisms was evaluated using the agar well diffusion method. ABS was tested on isolates obtained from 102 patients, including *Acinetobacter baumannii*, methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), extended-spectrum β-lactamase (ESBL) -producing *E. coli*, *Klebsiella* spp., *Acinetobacter* spp., *P. aeruginosa*, and S*tenotrophomonas maltophilia*. The zones of inhibition formed against ABS in both gram-negative and gram-positive bacteria ranged from 10–18 mm, indicating its antimicrobial effectiveness.[13]

Several studies have also investigated the antifungal activity of Ankaferd.[14-16] In a study by Akkoç et al., ABS subjected to the agar well diffusion test exhibited strong antifungal activity against *Zygosaccharomyces bailii*, *C. albicans*, *Mucor rouxii, Mucor brunnea, Aspergillus flavus,* and *Aspergillus parasiticus,* suggesting that Ankaferd possesses antifungal potential.[17] Similarly, in the study by Çiftçi et al.[18], ABS was added to the medium at different concentrations to assess its antifungal effects. As the concentration of Ankaferd increased, a greater inhibition of Candida isolates was observed. However, diluted forms of ABS did not inhibit fungal growth.

Our study examined the effects of Candida infection on liver, lung, and kidney histopathology in rats treated with ABS and those receiving saline. No significant histopathological differences were observed between the ABS and control groups in terms of antifungal activity in lung and kidney tissues. However, in liver tissue, a statistically significant difference was found in lymphoid infiltration and hepatocyte repair in the ABS group. The increased lymphoid infiltration in the ABS-treated group suggests that ABS may activate the immune system to enhance antifungal defense. This infiltration, which was more pronounced in ABS-treated rats, may indicate an immune response aimed at reducing the fungal load in liver tissue. However, further studies incorporating immunohistochemical analyses and serum cytokine level measurements are

required to elucidate this effect.

Additionally, the pronounced hepatocyte repair observed in the ABS group suggests that ABS may have tissue-regenerating properties. This effect is thought to be related to the antioxidant and anti-inflammatory properties of the herbal components in ABS. Literature data support the potential regenerative effects of Ankaferd on liver tissue. While the exact mechanism remains unclear, certain proteins are believed to mediate its anti-inflammatory and antioxidant effects.[19] For instance, in an experimental liver injury model by Satar et al.[20], minimal inflammation and complete regeneration were observed in the ABS-treated group, indicating promising histopathological changes. Similarly, in a study by Koşmaz et al.[21], ABS was shown to reduce inflammation, necrosis, and fibrosis in the liver of rats with obstructive jaundice. Moreover, in a study by Akbal et al., which investigated the gastrointestinal effects of high-dose oral ABS in rats, no gastrointestinal fibrosis, dysplasia, or metaplasia were observed, and histopathological examination of the liver showed no signs of inflammation, fibrosis, biliary destruction, or proliferation.[21] When evaluated together, the findings of lymphoid infiltration and hepatocyte repair in our study suggest that ABS initially activates the immune system to mount a defense against fungal infection. In the later stages, hepatocyte regeneration may be supported through inflammation-triggered regenerative mechanisms.

In the lung tissue, a higher frequency of inflammatory parameters such as neutrophilic leukocyte infiltration and edema, along with a lower incidence of intra-alveolar hemorrhage in the ABS group, may indicate the agent's potential immunomodulatory effects and its role in preserving vascular integrity. Despite these modest differences in inflammatory responses, histopathological findings in the kidney were similar between the groups. The absence of statistically significant differences may be attributed to the early-phase and mild nature of the tissue alterations, as well as the limited sample size within the study groups.

Despite the antimicrobial activity reported in in vitro studies, our study found no significant differences between the groups, except in liver tissue. This may be due to inadequate dosing of ABS or insufficient blood/serum concentrations. Additionally, gastrointestinal mucosal damage caused by chemotherapy in rats may have reduced the bioavailability of oral ABS. Furthermore, potential drug interactions between ABS and cyclophosphamide (used for neutropenia induction), amoxicillin, and polymyxin E (used to prevent secondary bacterial infections) might have limited its antifungal efficacy.

A major limitation of this study is the lack of quantitative assessment of *C. albicans* burden in the organs. Antifungal activity was evaluated solely based on histopathological findings. Future studies incorporating methods such as tissue culture or PCR to determine fungal load may provide a more accurate assessment of the antifungal efficacy of ABS

CONCLUSIONS

In conclusion, further comprehensive and well-controlled studies incorporating pharmacokinetic, pharmacodynamic, and quantitative analyses are needed to better elucidate the in vivo antimicrobial activity of ABS.

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Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Statement

Ethics committee approval was obtained from the İnönü University Faculty of Medicine Experimental Animals Ethics Committee (Protocol No: 2012/A-116, 27.12.2012).

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Authors' Contribution

Study Conception: IYN, FY; Study Design: IYN, FY; Materials: IYN, NŞ; Data Collection: IYN; Analysis and interpretion: IYN, FY, NŞ; Literature Review: IYN; Critical Review: IYN, FY, NŞ;

Manuscript preparing: IYN.

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