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# In Vitro Antifungal Activity of a Medicinal Plant Extract Mixture Against *Candida* Species Isolated from Patients with Oral Stomatitis

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## Abstract

**Background:** Ankaferd Blood Stopper® (ABS) is a hemostatic product comprising a standardized mixture of *Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum*, and *Urtica dioica*. It is used to control bleeding after extracorporeal injuries, traumatic cuts, dental operations, and surgical intervention. ABS was reported to exhibit antibacterial and germicidal activities.
**Objectives:** This in vitro study aimed to determine the antifungal activity of ABS.
**Methods:** In total, 114 *Candida* strains; 65 *Candida albicans* and 49 non-*albicans* isolated from the oral cavity of patients with oral stomatitis, as well as three reference strains of *C. albicans* ATCC 90028, *C.parapsilosis* ATCC 22019, and *C.krusei* ATCC 6258, were tested by agar well diffusion, disk diffusion, and time-kill curve methods in this study. The results obtained for ABS were compared with those obtained for amphotericin B (AMB).
**Results:** ABS and AMB exhibited inhibitory zones with mean diameters of 18.2 ± 1.4 (12-20) mm, 20.6 ± 1.2 (18 - 23) mm by disk diffusion and 18.3 ± 1.3 (15 - 20) mm, 19.9 ± 2.6 (18 - 22) mm, respectively, by agar well diffusion methods for *C. albicans*. On the other hand, ABS and AMB showed inhibition zones with mean diameters of 19.4 ± 1.5 (18 - 24) mm, 19.1 ± 2.8 (13 - 30) mm by disk diffusion and 19.8 ± 2.1 (18 - 25) mm, 18.7 ± 2.3 (13 - 23) mm by agar well diffusion methods for non-*albicans Candida* isolates. ABS exhibited higher activity against non-*albicans Candida* species compared to *C. albicans* ($P < 0.001$). By the time kill-curve method, ABS achieved a 4 $\log_{10}$ cfu/mL decrease in *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258, as well as seven different *Candida* spp. isolates of *C. albicans*, *C. glabrata, C. tropicalis, C. guilliermondii, C. kefyr, C. krusei*, and *C. parapsilosis*, respectively.
**Conclusions:** ABS can be an alternative for treating superficial infections.

*Keywords:* Ankaferd Blood Stopper, Antifungal Activity, *Candida*, Oral Stomatitis, Susceptibility

## 1. Background

Ankaferd Blood Stopper® (ABS) (Immun Ilaç Kozmetik Ltd. Sti., Istanbul, Turkey) is a hemostatic product consisting of a mixture of five plant extracts used in Turkish traditional medical applications. ABS comprises a standardized mixture of a dried leaf extract of *Thymus vulgaris* (5 g/100 mL), *Glycyrrhiza glabra* (9 g/100 mL), *Vitis vinifera* (8 g/100 mL), *Alpinia officinarum* (7 g/100 mL), and a dried root extract of *Urtica dioica* (6 g/100 mL). It is used to control bleeding after extracorporeal injuries, traumatic cuts, dental operations, and surgical intervention (1).

The mechanistic action of ABS is the formation of an encapsulated protein network formed by fibrinogen and blood proteins (such as ankyrin, spectrin, and actin), which provides focal points for the aggregation of erythrocytes. This protein network was observed to form in less than 1 s in vitro. In addition, ABS may be effective for individuals with a deficiency in hemostasis and for those with normal hemostatic parameters (2). Several clinical and experimental studies have reported the hemostatic effect of ABS (3-5).

ABS was also reported to exhibit antibacterial activity against Gram negative and Gram-positive bacteria (6-9) as well as mycobacteria (10), and germicidal (11) effect. However, there is no clarity as to whether ABS exhibits antifungal activity. To the best of our knowledge, two studies investigated the antifungal activity of ABS: Cinar et al. (12) reported that ABS inhibited the growth of *C. albicans* in vitro, while Ciftci et al. (13) did not detect any activity.

*Candida* species (spp.) are opportunistic pathogenic microorganisms that are found in the human oral cavity. Candidiasis is the most common fungal infection in the oral cavity and typically occurs by the abnormal prolifer-

ation of *Candida* spp. in the normal flora in the presence of factors such as immunosuppression, malnutrition, diabetes mellitus, hypothyroidism, malignancy, and the use of antibiotics and corticosteroids (14).

## 2. Objectives

The study aimed to determine the in vitro antifungal activity of ABS against *Candida* strains isolated from patients with oral stomatitis.

## 3. Methods

This study was designed as in vitro clinical experiments, exclusively focusing on yeast strains. These strains were obtained as a part of other studies conducted at the Istanbul Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkey during the past years. Clinical specimens were not collected from patients, and experiments were not conducted on humans and animals for the present study. Hence, the consent of the Ethics Committee is not required.

### 3.1. Clinical Candida Strains

In total, 114 *Candida* strains; 65 *Candida albicans* and 49 non-*albicans* (34 *C. glabrata*, six *C. tropicalis*, three *C. kefyr*, three *C. parapsilosis*, two *C. guilliermondii*, and one *C. krusei*) isolated from the oral cavity of patients with oral stomatitis were examined. Written consent was obtained from all patients. *Candida* isolates were stored at -80 degrees until testing. These isolates were subcultured onto Sabouraud dextrose agar (SDA) (Biolife, Milano, Italy) for analysis.

### 3.2. Antifungal Activity Assay

Standardized vials of the ABS (100 mL) were used in the experiments. The antifungal activity of ABS (Immun Ilac Kozmetik Ltd. Sti., Istanbul, Turkey) was evaluated by agar well diffusion (7, 15), disk diffusion (7, 16), and time-kill curve (13, 17) methods. For each isolate, standard inoculum suspensions were prepared in a sterile saline solution (8.5 g/L) (Sigma-Aldrich, Steinheim, Germany) from 24 hours cultures on SDA plates at 37°C and adjusted to the turbidity of a 0.5 Mc Farland standard solution ($10^6$ cfu/mL). *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258 were used as assay controls. The results obtained for ABS were compared with those obtained for amphotericin B (AMB) (Sigma, St. Louis MO, USA).

#### 3.2.1. Agar Well Diffusion Method

This experiment was conducted for the 114 strains isolated from patients, as well as the reference strains of *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258. Two wells with a diameter of eight mm were made on Mueller-Hinton agar (Merck, Darmstadt, Germany) supplemented with 2% glucose (Merck, Darmstadt, Germany) and 0.5$\mu$g/mL methylene blue (Himedia, Mumbai, India) under aseptic conditions. The standard inoculum suspension of the *Candida* isolate was spread on the agar. One well was filled with a 100 $\mu$L ABS, while the other well was filled with a 100 $\mu$L AMB solution (10 $\mu$g/100 $\mu$L). The petri plates were incubated at 37°C for 24 - 48 hours, followed by the measurement of the inhibition zones around the wells (7, 17).

#### 3.2.2. Disk Diffusion Method

This assay was performed as described in the Clinical Laboratory Standards Institute standard M44 A2 for all strains isolated from patients as well as the reference strains (16). The standard inoculum suspension of the isolate was swabbed over Mueller-Hinton agar supplemented with 2% glucose and 0.5 $\mu$g/mL methylene blue. Disks containing ABS (100 $\mu$L) and AMB (10 $\mu$g/100 $\mu$L) were prepared (Bioanalyse, Ankara, Turkey). For each isolate, an ABS disk and an AMB disk were placed on the agar surface. After the plates were incubated at 37°C for 24 - 48 hours, the inhibition zones were evaluated (7, 16).

#### 3.2.3. Time-Kill Curve Method

This method was performed for *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, and seven strains (*C. albicans*, *C. glabrata, C. tropicalis, C. guilliermondii, C. kefyr, C. krusei*, and *C. parapsilosis*) isolated from patients. For each isolate, three tubes containing 500 $\mu$L of the standard inoculum suspension of the isolate were prepared as mentioned above. One of these tubes was inoculated with 500 $\mu$L of ABS, the second one with 500 $\mu$L of the AMB solution (final concentration of 10 $\mu$g/100 $\mu$L), and the third one with a sterile saline solution (8.5 g/L) that was used as the positive control. All of the tubes were incubated at 37°C. Samples were immediately collected from these tubes and subsequently at 1, 6, 12, 24, and 48 hours after inoculation. A volume of 100 $\mu$L was removed from each tube and serial dilutions of $10^{-1}$, $10^{-2}$, and $10^{-3}$ were prepared using the sterile saline solution (8.5 g/L). Then, it was spread on SDA plates. After the plates were incubated at 37°C for 24 - 48 hours, the colonies were counted and expressed as colony forming units (cfu/mL) (13, 17).

### 3.3. Statistical Analysis

Statistical analysis was performed using the Statistical Analysis System Software, version 14.3 (SAS Institute Inc., Cary, NC, USA). The data were analyzed using the Mann-Whitney U and Kruskal-Wallis tests. The statistical significance level was set to 0.05.

## 4. Results

The antifungal activity of ABS was evaluated against 114 clinical (65 *C. albicans* and 49 non-*albicans*) (Figure 1) and three reference *Candida* strains (*C. albicans* ATCC 90028, C. parapsilosis ATCC 22019, *C. krusei* ATCC 6258) (Figure 2).

Table 1 summarizes the inhibition zones of ABS and AMB toward the reference *Candida* strains by agar well diffusion and disk diffusion methods.

By the agar well diffusion method, the mean diameters of the inhibition zones toward the clinical isolates were determined as 18.9 ± 1.8 (15 - 25) mm for ABS and 19.4 ± 2.5 (13 - 23) mm for AMB. By the disk diffusion method, the mean diameters of the inhibition zones were 18.7 ± 1.5 (12 - 24) mm for ABS, and 19.9 ± 2.1 (13 - 30) mm for AMB.

Table 2 summarizes the mean and median of the inhibition zones of ABS and AMB toward the clinical *C. albicans* and non-*albicans Candida* isolates by agar well and disk diffusion methods. In this study, for *C. albicans*, ABS and AMB exhibited inhibition zones with mean diameters of 18.2 ± 1.4 (12 - 20) mm, 20.6 ± 1.2 (18 - 23) mm by disk diffusion, and 18.3 ± 1.3 (15 - 20) mm, 19.9 ± 2.6 (18 - 22) mm by agar well diffusion methods, respectively. In case of non-*albicans Candida* isolates, ABS and AMB exhibited inhibition zones with mean diameters of 19.4 ± 1.5 (18 - 24) mm, 19.1 ± 2.8 (13 - 30) mm by disk diffusion, and 19.8 ± 2.1 (18 - 25) mm, 18.7 ± 2.3 (13 - 23) mm by agar well diffusion methods, respectively. The comparison of these results revealed that ABS exhibits showed higher activity against non-*albicans Candida* species compared to *C. albicans* ($P < 0.001$).

By the time kill-curve method, ABS was tested and found to be active against *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, and the seven clinical *Candida* spp. isolates of *C. albicans*, *C. glabrata, C. tropicalis, C. guilliermondii, C. kefyr, C. krusei*, and *C. parapsilosis*, respectively. ABS exhibited fungicidal activity with a 4 $\log_{10}$ cfu/mL decrease compared to the starting inoculum of all of the tested *Candida* isolates (Table 3).

## 5. Discussion

Previously, ABS was reported to exhibit activity against bacteria including the most frequent causes of nosocomial antibiotic resistant infections, such as *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia, Enterobacter* spp., vancomycin-resistant *Enterococcus*, methicillin-resistant *Staphylococcus aureus*, and mycobacteria (6-10). In this study, the antifungal activity of ABS was evaluated against 114 clinical and three reference *Candida* strains by agar well diffusion, disk diffusion, and time-kill curve methods. Agar well diffusion is widely employed for investigating the in vitro antimicrobial activity of various chemicals (6, 7, 15). The broth microdilution assay could not be performed as the detected ABS did not dissolve in broth media as reported in other studies (7, 12). The activity of ABS was compared to that of AMB.

ABS exhibited activity against all the tested isolates including reference and clinical strains. By the agar well diffusion method, the mean diameters of the inhibition zones were 18.9 ± 1.8 (15 - 25) mm for ABS and 19.4 ± 2.5 (13 - 23) mm for AMB. By the disk diffusion method, the mean diameters of the inhibition zones were 18.7 ± 1.5 (12 - 24) mm for ABS and 19.9 ± 2.1 (13 - 30) mm for AMB. No statistical difference was observed between the results obtained by these two methods ($P = 0.356$).

By the time kill-curve method, ABS exhibited activity against *C. albicans* ATCC 90028, C. parapsilosis ATCC 22019, C. krusei ATCC 6258, and seven clinical *Candida* spp. isolates of *C. albicans*, *C. glabrata, C. tropicalis, C. guilliermondii, C. kefyr, C. krusei*, and *C. parapsilosis*, respectively. Compared to the starting inoculum, ABS exhibited fungicidal activity against all of the tested *Candida* isolates. ABS exhibited a 4 $\log_{10}$ cfu/mL decrease in *C. glabrata*, *C. guilliermondii*, and *C. kefyr* by the end of the 6th; *C. tropicalis* by the end of the 12th; *C. parapsilosis, C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258 by the end of the 24th; *C. albicans* clinical isolate, *C. albicans* ATCC 90028, and *C. krusei* by the end of the 48th hour of application.

As mentioned previously, only two studies reported the antifungal activity of ABS. Cinar et al. (12) investigated the effect of ABS compared to 0.2% chlorhexidine digluconate (CHX), and ferric sulphate (FS) on various oral microorganisms including *C. albicans* (one oral isolate and ATCC 10231) by the agar well diffusion method. The authors reported that although ABS exhibits antifungal activity against the oral isolate and the reference strain, inhibition zones (7.39 and 5.79 mm) were less than those obtained for FS (16.85 and 14.71 mm) and CHX (13.95 and 11.03 mm). Ciftci et al. (13) evaluated the antifungal activity of different volumes of ABS only for four *C. albicans* isolates consisting of ATCC 90028 and three clinical isolates from the oral cavity by the agar diffusion and broth dilution methods. The authors also investigated the effect of ABS over time. By the agar diffusion test, the diameters of the inhibition zones increase with the increase in the ABS volume. MIC values were not

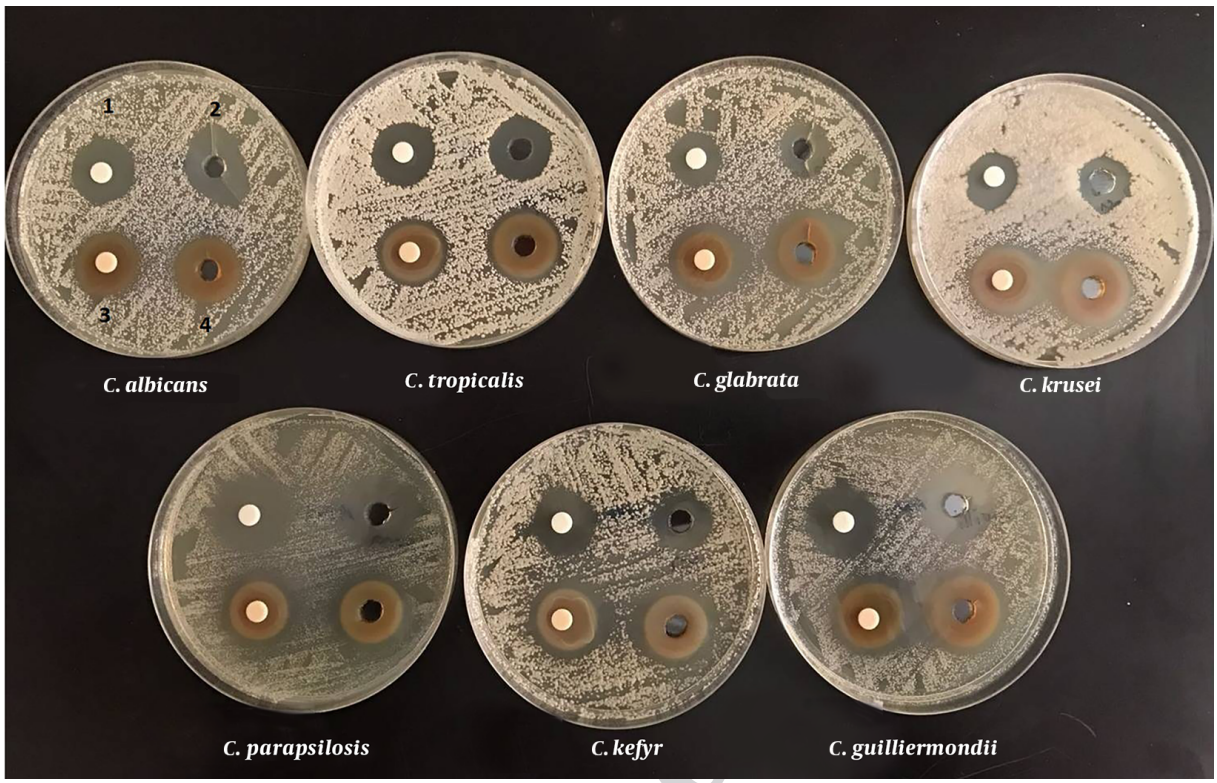


**Figure 1.** Inhibition zones obtained for clinical isolates. 1, Disk diffusion method for amphotericin B (AMB); 2, Agar well diffusion method for AMB; 3, Disk diffusion method for Ankaferd Blood Stopper (ABS); 4, Agar well diffusion method for ABS.

**Table 1.** Inhibition Zones for Standard *Candida* Isolates

| Isolate | Disc Diffusion Method, mm | | Agar Well Diffusion Method, mm | |
|---|---|---|---|---|
| | AMB | ABS | AMB | ABS |
| ***C. albicans* ATCC 90028** | 18 | 19 | 18 | 19 |
| ***C. parapsilosis* ATCC 22019** | 19 | 19 | 19 | 19 |
| ***C. krusei* ATCC 6258** | 15 | 24 | 16 | 25 |

**Table 2.** Inhibition Zones for Clinical *C. albicans* and Non-*albicans Candida* Isolates

| Methods | Inhibition Zones (mm) | | | | | | P Value |
|---|---|---|---|---|---|---|---|
| | *C. albicans* | | | Non-*albicans Candida* | | | |
| | Mean ± SD (Min - Max) | Median | 25 - 75 Percentiles | Mean ± SD (Min - Max) | Median | 25 - 75 Percentiles | |
| **Disc diffusion** | | | | | | | |
| AMB | 20.6 ± 1.2 (18 - 23) | 20 | 20 - 22 | 19.1 ± 2.8 (13 - 30) | 20 | 18 - 20 | < 0.001 |
| ABS | 18.2 ± 1.4 (12 - 20) | 18 | 18 - 18 | 19.4 ± 1.5 (18 - 24) | 20 | 18 - 20 | < 0.001 |
| **Agar well diffusion** | | | | | | | |
| AMB | 19.9 ± 2.6 (18 - 22) | 20 | 20 - 20 | 18.7 ± 2.3 (13 - 23) | 20 | 18 - 20 | 0.001 |
| ABS | 18.3 ± 1.3 (15 - 20) | 18 | 18 - 19 | 19.8 ± 2.1 (18 - 25) | 20 | 18 - 20 | < 0.001 |

determined by the broth dilution method because of the coagulation of the substances in the broth. However, the authors did not observe any significant effect after incubating the reference *Candida* strain with different ABS concentrations for 10, 20, and 40 min. Hence, ABS did not exhibit antifungal activity.

The result obtained in this study is in agreement with that reported by Cinar et al (12). Antifungal activity was

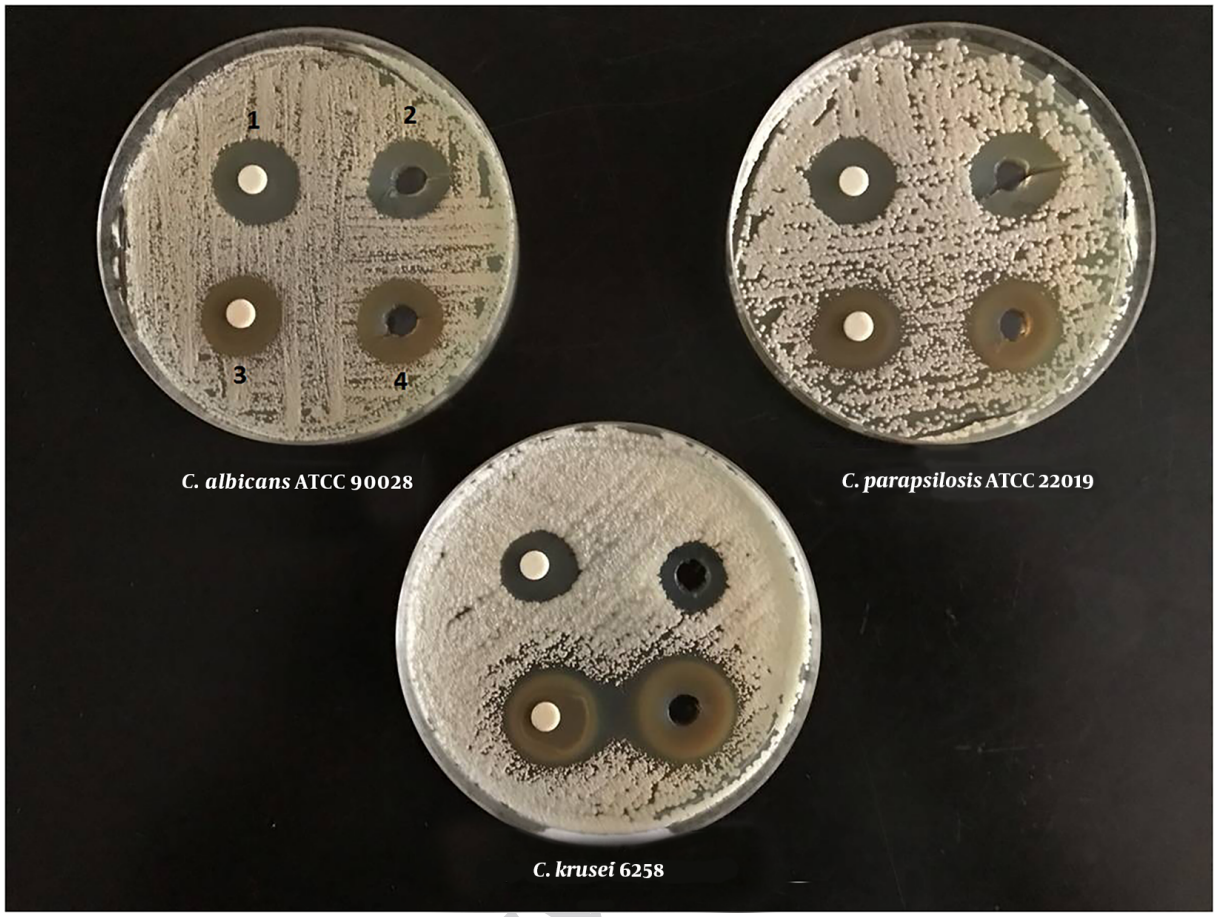


**Figure 2.** Inhibition zones obtained for standard strains. 1, Disk diffusion method for amphotericin B (AMB); 2, Agar well diffusion method for AMB; 3, Disk diffusion method for Ankaferd Blood Stopper (ABS); 4, Agar well diffusion method for ABS.

**Table 3.** Results Obtained with Ankaferd Blood Stopper by Time-kill Curve Method

| Isolates | 0 h | | 1 h | | 6 h | | 12 h | | 24 h | | 48 h | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | ABS | C | ABS | C | ABS | C | ABS | C | ABS | C | ABS |
| *C. albicans* | 120[a] | 131 | 130 | 101 | 112 | 101 | 108 | 92 | 153 | 6 | 280 | 0 |
| *C. glabrata* | 240 | 253 | 201 | 150 | 198 | 0 | 206 | 0 | 249 | 0 | 284 | 0 |
| *C. tropicalis* | 225 | 272 | 212 | 5 | 296 | 1 | 207 | 0 | 215 | 0 | 290 | 0 |
| *C. guilliermondii* | 270 | 281 | 192 | 158 | 201 | 0 | 215 | 0 | 258 | 0 | 292 | 0 |
| *C. kefyr* | 188 | 182 | 165 | 72 | 174 | 0 | 185 | 0 | 220 | 0 | 263 | 0 |
| *C. krusei* | 142 | 164 | 151 | 95 | 108 | 21 | 132 | 11 | 155 | 6 | 256 | 0 |
| *C. parapsilosis* | 201 | 218 | 192 | 110 | 179 | 14 | 201 | 1 | 228 | 0 | 270 | 0 |
| *C. albicans* ATCC 90028 | 152 | 131 | 140 | 99 | 117 | 78 | 132 | 18 | 174 | 3 | 310 | 0 |
| *C. parapsilosis* ATCC 22019 | 143 | 129 | 122 | 100 | 113 | 11 | 136 | 6 | 152 | 0 | 210 | 0 |
| *C. krusei* ATCC 6258 | 160 | 142 | 172 | 95 | 126 | 33 | 137 | 11 | 148 | 0 | 227 | 0 |

Abbreviations: ABS, Ankaferd Blood Stopper; C, control; h, hours.
[a] $\times 10^4$ cfu/mL.

probably not detected in the study by Ciftci et al. (13) because of the short incubation times of 10, 20, and 40 min. In this study, a 4 $\log_{10}$ cfu/mL decrease was detected the earliest at the end of the 6th hour in the *C. glabrata*, *C. guillier-*

*mondii*, and *C. kefyr* strains isolated from patients.

To the best of our knowledge, this is the first study that examines the antifungal activity against the highest number of isolates as well as non-*albicans Candida* isolates. In this study, for *C. albicans*, ABS and AMB exhibited inhibition zones with mean diameters of 18.2 ± 1.4 (12 - 20) mm, 20.6 ± 1.2 (18 - 23) mm by disk diffusion and 18.3 ± 1.3 (15 - 20) mm, 19.9 ± 2.6 (18 - 22) mm by agar well diffusion methods, respectively. For the non-*albicans Candida* isolates, ABS and AMB exhibited inhibition zones with mean diameters of 19.4 ± 1.5 (18 - 24) mm, 19.1 ± 2.8 (13 - 30) mm by disk diffusion and 19.8 ± 2.1 (18 - 25) mm, 18.7 ± 2.3 (13 - 23) mm by agar well diffusion methods, respectively. ABS was confirmed to exhibit higher activity against non-*albicans Candida* species than *C. albicans* ($P < 0.001$).

In conclusion, ABS exhibits antifungal activity against *Candida* isolates. It exhibits higher activity against non-*albicans Candida* isolates than *C. albicans*. ABS may be an alternative particularly for superficial infections. Nevertheless, further studies need to be carried out for examining the use of ABS as an alternative antifungal agent.

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## Footnotes

**Authors' Contribution:** Study concept and design: Ozcan Erdogan, Ozgul Kisa, and Dilek Satana; conducting the experiments: Gonca Erkose Genc, Ozcan Erdogan, Candan Demir; data analysis and interpretation of results: Gonca Erkose Genc, Ozcan Erdogan, Candan Demir, Ozgul Kisa, and Dilek Satana; drafting of the manuscript: Gonca Erkose Genc, Dilek Satana; supervising the study: Ozgul Kisa, Dilek Satana. All authors revised and approved the final manuscript.

**Conflict of Interests:** Authors had no conflict of interests.

**Ethical Considerations:** This study was designed as in vitro clinical experiments, exclusively focusing on yeast strains. These strains were obtained as a part of other studies conducted at the Istanbul Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkey during the past years. Clinical specimens were not collected from patients, and experiments were not conducted on humans and animals for the present study. Hence, the consent of the ethics committee is not required.

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