



In vitro Antifungal Activities of Fluconazole, *Camellia sinensis* and *Cydonia oblonga* Leaf Extracts Against *Candida* Species Isolated from Blood Cultures

Kan Kültürlerinden İzole Edilen *Candida* Türlerinde Flukonazol, *Camellia sinensis* ve *Cydonia oblonga* Yaprak Ekstraktlarının *In vitro* Etkinliği

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ABSTRACT

Objective: In this study we investigated *in vitro* antifungal activity of fluconazole, green tea (*Camellia sinensis*) and quince leaf (*Cydonia oblonga*) extracts in *Candida* strains isolated from blood cultures.

Methods: Fifty *Candida* spp were included to study. *Camellia sinensis* and *Cydonia oblonga* leaves collected from Rize and Erzurum/Tortum regions were prepared as an extract. Then stock solutions of the extracts and fluconazole powder obtained from the manufacturer were prepared for use in the Broth Microdilution (BMD) method according to Clinical and Laboratory Standards Institute (CLSI) M27 A3 recommendation. Fluconazole minimum inhibitory concentration (MIC) values obtained from BMD method were evaluated according to CLSI M27 A4 break points. MIC values obtained for *Camellia sinensis* and *Cydonia oblonga* were interpreted according to fluconazole break points since there is no break points in the literature.

Results: Of the 50 *Candida* spp. included in the study, 37 (74%) were *C. albicans* while 13 (26%) were non-*albicans* species. The most common isolated non-*albicans* species was *C. parapsilosis* (14%). For all tested *Candida* strains MIC₅₀ and MIC₉₀ values for fluconazole were found to be 0.125 µg/mL- 0.25 µg/mL while they

ÖZ

Amaç: Çalışmada kan kültürlerinden izole edilen *Candida* suşlarında yeşil çay (*Camellia sinensis*) ve ayva yaprağı (*Cydonia oblonga*) ekstraktları ile flukonazolün *in vitro* etkinliği araştırılmıştır.

Yöntemler: Çalışmaya 50 *Candida* spp. dahil edildi. Rize ve Erzurum/Tortum bölgelerinden toplanmış *Camellia sinensis* ve *Cydonia oblonga* yaprakları ekstrakt olarak hazırlandı. Daha sonra üretici firmadan elde edilen flukonazol toz etken maddesi ile hazırlanan ekstraktlarından CLSI M27 A3 önerileri doğrultusunda Broth Mikrodilüsyon (BMD) yönteminde kullanılmak üzere stok solüsyonlar hazırlandı. BMD yönteminden elde edilen flukonazol MİK değerleri CLSI M27 A4 sınır değerlerine göre değerlendirilirken *Camellia sinensis* ve *Cydonia oblonga* için elde edilen MİK değerleri literatürde sınır değerler bulunmadığından flukonazol sınır değerlerine göre yorumlandı.

Bulgular: Çalışmaya dahil edilen 50 *Candida* spp.'nin 37'si (%74) *C. albicans* iken 13'ü (%26) non-*albicans* idi. Non-*albicans* türleri içerisinde en sık izole edilen tür *C. parapsilosis* idi (%14). Flukonazol için MIC₅₀ ve MIC₉₀ sırasıyla 0,125 µg/mL- 0,25 µg/mL iken, aynı

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were determined as 0.125 µg/mL-64 µg/mL and 0.125 µg/mL-0.125 µg/mL for green tea and quince leaves, respectively.

Conclusion: According to the low MIC values, it is seen that green tea and quince leaf are *in vitro* effective against *Candida species*. However, further extensive *in vitro* and *in vivo* studies should be done on the possibility that green tea and quince leaf can be used alternatively in the treatment of invasive *Candida* infections.

Keywords: Antifungal activity, *Candida*, fluconazole, green tea, quince leaf

değerler *Camellia sinensis* için 0,125µg/mL-64 µg/mL; *Cydonia oblonga* için 0,125 µg/mL-0,125 µg/mL olarak tespit edildi.

Sonuç: Elde edilen düşük bir MİK değeri dikkate alındığında yeşil çay ve ayva yaprağının invazif *Candida* enfeksiyonlarının tedavisinde alternatif olarak kullanılabileceği düşünülmüştür.

Anahtar Sözcükler: Antifungal etkinlik, *Candida*, flukonazol, yeşil çay, ayva

Introduction

Invasive *Candida* infections are one of the major health problems with high mortality and morbidity rates, increasing hospitalization and cost of treatment (1). The incidence of candidemia cases is increased by malignancies, immunosuppressive diseases or treatments, use of broad-spectrum antibiotics and corticosteroids, aggressive chemotherapy and some major surgical procedures all over the world (2,3). According to the Centers for Diseases Control and Prevention (CDC) and the National Healthcare Safety Network (NHSN), *Candida* species are the fourth most common sepsis cause in America and Europe (2,4-6).

Azoles are often used in the treatment of fungal infections with the advantage that they have less toxic effects than other antifungal agents. However, this situation recently has caused increased resistance to azole in fungal agents which prompts researchers to search for new antifungal agents, especially for resistant invasive *Candida* infections (7,8).

Green tea (*Camellia sinensis*) and quince (*Cydonia oblonga*) leaf have been used for treatment of various infections in traditional medicine for centuries. And also there are various studies on antimicrobial activity of green tea and quince leaf in the literature most of which showed that green tea and quince leaf both have *in vitro* antimicrobial effects (9-12).

In this study, we investigated *in vitro* antifungal activity of green tea and quince leaf on *Candida* isolates obtained from blood cultures. The minimum inhibitory concentration (MIC) values of fluconazole were also evaluated for each isolates.

Methods

Candida strains: Fifty *Candida* spp. isolated from blood cultures (BACTEC 9000 System; Becton Dickinson) and sent to Medical Microbiology Laboratory between April 2013-December 2015 were included to the study. Repeated samples of the same patient were not included in the study. Chromogenic agar, tween 80 agar and related typing cards of VITEK 2 automatized system (BioMerieux, France) were used to identify the isolated *Candida species*. Typed strains were stored at -80°C with stock preparations as far as the planned schedule of the study. The strains were passaged twice in Sabouraud Dextrose Agar (Oxoid, UK) after dissolved at room temperature to obtain appropriate culture. In the study, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 strains were used as control strains.

Preparation of extracts: Quince (*Cydonia oblonga*) and green tea (*Camellia sinensis*) leaves were collected from Tortum/Erzurum and Rize in their appropriate season. The dried and powdered leaves (100 g) of green tea and quince were extracted with 1 L of methanol using a Soxhlet extractor (SIGMA-ALDRICH, 322415) for 72 h at a temperature which was not exceeding the boiling point of the solvent. The extract was filtered using Whatman filter paper No.1 (Dassel, Germany) and then concentrated in vacuo at 40°C using a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland). The plant extracts were then lyophilized and kept at 4°C until being tested (13).

Preparation of stock solutions: While fluconazole powder (Pfizer) was dissolved and diluted in sterile, pyrogen-free saline to a stock concentration of 256 µg/mL according to CLSI M27-A3 suggestions (14), prepared green tea and quince leaf extracts were both diluted to the concentration of 256 µg/mL as same as fluconazole since there is no standard MIC values for quince leaf and green tea extracts.

Broth Microdilution (BMD) method: To obtain MIC values of fluconazole, green tea and quince leaf for *Candida* strains, BMD method was applied by using L-glutamine, bicarbonate free, RPMI 1640 medium (Sigma Chemical Co., St Louis, Mo., USA) according to CLSI M27-A3 suggestions (14). This medium was used in the BMD test after adding 2% dextrose and adjusting pH to 7 with 0.165 M morpholine-propane-sulfonic acid (MOPS; Sigma). In the direction of this method two-fold decreasing serial dilutions of fluconazole, green tea and quince leaf were prepared in sterile, U-based, 96 well microplates by using stock solutions. Then *Candida* strains adjusted to 0.5 McFarland standard turbidity were added in each well of microplates except the last one. The last two wells were separated as sterilization control and reproduction control wells. Thus, the final concentration of fluconazole green tea and quince leaf were between range of 64 µg/mL to 0.125 µg/mL. Plates were evaluated by two different investigators with naked eyes after 24-48 hours of incubation. The MIC value of the well with the smallest amount of drug with 50% or less reproduction relative to the reproduction control was recorded as MIC value. Fluconazole MIC values were evaluated according to the CLSI M27- S4 break points (15). Because of there is no standard MIC break points for quince leaf and green tea extracts, MIC values were evaluated by comparing with fluconazole break points.

Statistical Analysis

For analysis of the study data Statistical Package for Social Sciences (SPSS; v20.0) statistical program was used. The data were expressed as number, percentage, mean and standard deviation. Suitability to normal distribution of the data was investigated by the Kolmogorov-Smirnov test. The Kruskal Wallis test was used for the comparison of more than two groups of numerical data without normal distribution. The results were considered significant when $p < 0.05$.

Results

Total of 50 *Candida* spp (37 *C. albicans*, 7 *C. parapsilosis*, 3 *C. tropicalis*, 3 *C. glabrata*) were included in the study. *Albicans* species (74%) were found to be more than *non-albicans* species (26%). The most common isolated *non-albicans* species was *C. parapsilosis* (14%).

Candida strains isolated from blood cultures of hospitalized patients in 11 different clinics in our hospital were included in the study. The clinics where these strains were most isolated were Anesthesia and Reanimation Intensive Care Unit (22%), Internal Medicine Intensive Care Unit (16%), Internal Medicine Hematology Service (12%), Pediatric Intensive Care Unit (12%) and Medical Oncology Service (10%), respectively. Distribution

of isolated *Candida* strains according to the services are shown in the Table 1.

For all tested *Candida* strains MIC₅₀ and MIC₉₀ values for fluconazole were found to be 0.125 µg/mL-0.25 µg/mL while they were determined as 0.125 µg/mL-64 µg/mL and 0.125 µg/mL-0.125 µg/mL for green tea and quince leaves, respectively. There was no significant difference between fluconazole, green tea and quince leaf extract values obtained from *Candida* strains.

When MIC values were evaluated according to CLSI M27-S4 break points, all of the *C. tropicalis* and *C. glabrata* strains were susceptible; 2 *C. parapsilosis* and 1 *C. albicans* were resistant to fluconazole (MIC=32 µg/mL). The MIC values of green tea and quince leaves for fluconazole resistant strains were 64 µg/mL. The results of MIC₅₀, MIC₉₀ and MIC range obtained with BMD method against *Candida* strains for fluconazole, green tea and quince leaf are shown in the Table 2.

Discussion

Candida spp are one of the important causes of nosocomial bloodstream infections. The incidence of *Candida* bloodstream infections have risen in the past 20 years worldwide. Generally *C. albicans* is the most frequently isolated *Candida* species in blood cultures, however in some studies *non-albicans* species have been

Table 1. Distribution of isolated *Candida* strains according to the services

Services	<i>C.albicans</i>	<i>C.parapsilosis</i>	<i>C.tropicalis</i>	<i>C.glabrata</i>	Total
Anesthesia/Reanimation ICU*	7	3	1	-	11
Internal Medicine ICU	6	1	-	1	8
Internal Medicine Hematology	4	1	-	1	6
Pediatric ICU	5	-	1	-	6
Medical Oncology	5	-	-	-	5
Neurology ICU	3	1	-	-	4
Organ Transplant	2	-	1	1	4
Infectious Diseases	2	-	-	-	2
Pediatric Hematology	1	1	-	-	2
Cardiology ICU	1	-	-	-	1
Brain Surgery ICU	1	-	-	-	1
Total	37	7	3	3	50

*ICU: Intensive Care Unit

Table 2. The results of MIC₅₀, MIC₉₀ and MIC_{min-max} obtained with BMD method against *Candida* strains for fluconazole, green tea and quince leaf (µg/mL)

Strains	MIC ₅₀			MIC ₉₀			MIC _{min-max}		
	Flu	GT	QL	Flu	GT	QL	Flu	GT	QL
<i>C.albicans</i> (37)	0.125	0.125	0.125	0.25	2	0.125	0.125-32	0.125-64	0.125-64
<i>C.parapsilosis</i> (7)	0.125	0.125	0.125	32	64	64	0.125-32	0.125-64	0.125-64
<i>C.tropicalis</i> (3)	-	-	-	-	-	-	0.125-0.25	0.125-0.25	0.125-0.25
<i>C.glabrata</i> (3)	-	-	-	-	-	-	0.125-0.25	0.125-0.25	0.125-0.25
All strains	0.125	0.125	0.125	0.25	64	0.125	0.125-32	0.125-64	0.125-64

*Flu: Fluconazole, GT: Green tea leaf, QL: Quince leaf, min: Minimum, max: Maximum, BMD: Broth microdilution

isolated more frequently. The distribution of isolated species may vary by region. The most common *non-albicans* species isolated from blood cultures are *C. parapsilosis*, *C. glabrata* and *C. tropicalis*. It has been reported that *C. krusei* is the least frequently isolated species (16-19). In our study *C. albicans* was the most frequently isolated species (74%) while *C. parapsilosis* was the most common among *non-albicans* species.

Today we use various antifungal agents, such as azoles, that raised significant progress in treatment of invasive *Candida* infections. Fluconazole is very often used with the advantage of its broad spectrum and low toxicity than others. Although in our study, fluconazole resistance was 6% (2 *C. parapsilosis* and 1 *C. albicans*), some studies showed that azole resistance have increased recently because of common use (20-23). Due to this current case and inadequate antifungal treatment options, *Candida* bloodstream infections are still common extending the length of stay in hospitals and increasing the cost of care (24,25). That makes researchers look for alternative antifungal agents. There are many plants with medical features. There is a widespread belief that medicinal plants are healthier and more harmless or safer than synthetic ones. These medicinal plants have a rich content that can be used to develop drugs. In recent years, studies on antifungal properties of some plants have been revealed (26-28).

Green tea is an easily accessible plant in Turkey. Previous studies have given positive results on antifungal activity of green tea. According to the results of the studies, in the future the green tea extracts may be considered as a therapeutic adjunct on oral *Candida* species (10,29,30). In a study, it was demonstrated that green tea was found to be very effective on *C. albicans*, *Streptococcus mutans* and *Lactobacilli* spp (31). Yang and Jiang (32) have reported that tea polyphenols can inhibit mycelial growth and spore germination of *Rhizopus stolonifer*. Aladag et al. (33) demonstrated that the antifungal activity of green tea leaves may be closely correlated to catechin content which can vary due to different harvest time. In this study fresh, green tea leaves were harvested in May which was the most convenient time and the MIC₅₀ and MIC₉₀ values were found to be 0.125 µg/mL-64 µg/mL. According to these results, it is interpreted that green tea is *in vitro* effective on *Candida* strains isolated from blood culture.

Quince, which is the other plant we used in this study (*Cydonia oblonga*), belongs to the family *Rosaceae* and Turkey is one of the most important quince producers worldwide (34). Leaf, fruit and seed of quince have very important medicinal effects. In studies with quince, many useful features have been reported such as antibacterial, antioxidant and antiproliferative, antiinflammatory and anti-dysenteric activities. Furthermore, it has been reported that it can also be useful in cancer, bloodstream and skin diseases (27). Fattouch et al. (35) have studied with various parts of quince and reported that quince may be a useful adjuvant agent for the treatment of bacterial infections in addition to antibiotics. Alizadeh et al. (26) reported that the quince leaf's extracts can inhibit the growth of *Aspergillus niger*. However, there is no satisfactory research result on anticandidal properties of quince leaf in the literature. In this study, we have investigated

anticandidal effects of quince leaf's methanolic extracts on 50 *Candida* species. The MIC₅₀ and MIC₉₀ values were 0.125 µg/mL-0.125 µg/mL, respectively and these results indicated that quince leaf extracts have good *in vitro* anticandidal effects with very low MIC levels.

Although the MIC₅₀ and MIC₉₀ values of both green tea and quince leaf extracts were very low, the situation was different in fluconazole resistant strains. In our study, 2 *C. parapsilosis* and 1 *C. albicans* strains were resistant to fluconazole and fluconazole MIC values were 32 µg/mL for all of them. When the MIC values of green tea and quince leaf of these strains were examined, it was found that the MIC value was 64 µg/mL for these strains. Because of this result, it was thought that green tea and quince leaf were not effective in fluconazole resistant *Candida* strains.

There are various studies on the use of green tea and quince leaf for therapeutic purposes and their effects on the body. In some of these studies the distribution of green tea components were calculated in blood and urine after oral intake (36). These studies serve as guides to show the distribution of green tea to the part of the body that is infected. One of these studies showed that, a cup of Japanese green tea contained about 150 mg of epigallocatechin and about 3.5 mg of it excreted into the urine per cup of tea (37). There are also many studies showing the phenolic components of quince leaf and the therapeutic effects of these components on the body (27,38,39). Though these studies include only oral use, they are hopeful that green tea and quince leaf contents can be used to develop different form of drugs.

Study Limitations

In this study, the phenolic profile of the green tea and quince leaf extracts could not be analyzed. Since the study was conducted with *Candida* strains isolated from the blood culture samples which were sent to our laboratory in a specified time period, no preliminary statistical study was done in terms of the number and types of strains to be used in the study.

Conclusion

In addition to making contributions to the literature about the antifungal activity of green tea on *Candida* species, this is the first study directly conducted on the evaluation of antifungal activity of quince leaf extracts on *Candida* species isolated from blood culture. According to the low MIC values obtained from the study, it was observed that green tea and quince leaf extracts both have *in vitro* antifungal activity on *Candida* species. However, further extensive *in vitro* and *in vivo* studies should be undertaken whether green tea and quince leaf can be used in the treatment of invasive *Candida* infections.

Ethics

Ethics Committee Approval: This study was approved by the Ethics Committee of Faculty of Medicine of Ataturk University, dated 28.04.2016 and numbered 4/08.

Informed Consent: Written informed consent was not received due to the nature of this study.

Peer Review: Externally peer-reviewed.

Authorship Contributions

Concept: H.H., Design: H.H., M.V.C., S.Ş., Data Collection or Processing: H.H., M.V.C., Analysis or Interpretation: H.H., M.V.C., Literature Search: H.H., M.V.C., H.İ., Writing: H.H., M.V.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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