Original Article

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In-vitro Antifungal Activities of Kombucha Tea Culture Supernatant Combined with Voriconazole against Vulvovaginal Candidiasis Clinical Isolates

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Abstract:

Objective: To investigate the antifungal activity of voriconazole, with and without Kombucha tea culture, against *Candida* strains isolated from vulvovaginal candidiasis.

Material and Methods: The study included 150 females, within child-bearing periods, complaining of valvovaginal candidiasis. *Candida* strains were isolated, and identified by conventional microbiological methods; and confirmed by Viteck-2 System. The sensitivity of the isolates to voriconazole was performed, via the Disc diffusion method. Resistant strains were then subjected to minimum inhibitory concentrations (MIC) investigation of voriconazole alone, and in combination with a Kombucha tea culture via the broth micro-dilution method in concentrations ranging from 0.0048 to 10 µg/ml. The ability of voriconazole, with and without Kombucha, to eradicate *Candida* biofilms were investigated using a crystal violet absorbance assay.

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Results: Eighty-nine strains were isolated. From these 60 isolates showed variable resistance patterns (57 were voriconazole resistant, and 3 had dose-dependent susceptability). Kombucha significantly decreased the MIC_{50} of voriconazole against all strains from 5 to 0.625 µg/ml (p-value<0.01); additionally, MIC_{90} were reduced from 10 to 1.25 µg/ml (p-value =0.000). Voriconazole at a concentration of 0.156 µg/ml succeeded in eradicating biofilms formed by 18 strains after adding a Kombucha tea supernatant versus zero strains when using Voriconazole alone.

Conclusion: Kombucha Black tea cultures could be promising antifungal agents in the treatment of vulvovaginal candidiasis.

Keywords: antimicrobial, biofilm, Kombucha tea culture, voriconazole, vulvovaginal candidiasis

Introduction

Candida species are present in the vagina as a part of vaginal microbiota, and usually do not lead to diseases. The interactions and synergies among the host microbial flora in the vagina are responsible for maintenance of the balance in the vaginal environment[1]. Valvovaginal candidiasis (VVC) is reported to be associated with the presence of various risk factors; such as, individual susceptibility, increased frequency of sexual intercourse, prolonged antibiotic therapy, administration of contraceptive pills, spermicide use, diabetes, pregnancy and immunosuppression[2,3]. Although, *Candida albicans* is the most common cause of VVC, other species have also been implicated with increased incidence of infection; such as, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei*[4-6]. Increased incidences of VVC, by other *Candida* species, is contributed to high rates of resistance and recurrence[4,7]. Azoles are commonly used to treat VVC; however, the problem when using antifungals, in addition to safety and cost, is the development of resistant strains[8]. Currently, there is a rising evidence of increased azole resistance between isolates of the *Candida* species from VVC, other than *Candida albicans*; as they are generally more resistant to azoles, than *C. albicans*[9]. Previous reports have highlighted that antifungals were inappropriately prescribed in cases with negative culture. The misuse of antifungals contributes to the development of resistance, and also the emergence of infections associated with other *Candida* species as well as other opportunistic fungi[10]. Prolonged therapies and increased usage of antifungal therapy for recurrent candidiasis are the most common causes of azole resistance among *Candida* isolated from vulvovaginal candidiasis. Most non-*C. albicans* species have higher, minimum inhibitory concentrations (MICs) to azoles antifungals, and so the infections they cause are difficult to be treated[11]. This phenomenon emphasizes the importance in the identification of new antifungal agents that have good potency against resistant *Candida* strains, with low side effects.

Probiotics are harmless, live microorganisms that improve many nutritional and digestive functions[12]. They are reported to be safe, and have many beneficial effects in addition they can also be used as therapies for various diseases. Kombucha is a beverage of sweetened black tea, fermented with a symbiotic combination of yeasts and acetic acid bacteria[13]. Kombucha has been found to contain metabolites with antimicrobial activity; such as catechins and acetic acid, in addition to antibiotic substances that inhibit Gram-positive and Gram-negative microorganisms[14]. Kombucha has antimicrobial activity against standard *Candida* strains; such as, *C. albicans* CCM 8186, *C. glabrata CCM 8270, C. krusei CCM 8271, C. tropicalis CCM 8223 and bacterial strains; including: H. influenzae CCM 4454, E. coli CCM 3954, S. aureus ATCC 25923, S. epidermidis*

CIP 106510, M. luteus NCIMB 8166, S. typhimurium LT2, E. coli ATCC 35218, P. aeruginosa ATCC 27853 and *L. monocytogenes ATCC 19115*[15,16]

The aim of this present study was to investigate the antifungal activity of voriconazole with and without Kombucha tea culture supernatant against clinical *Candida* strains isolated from vulvovaginal candidiasis.

Material and Methods

Isolation of *Candida* strains

This study was conducted from September 2019 to March 2021, and included 150 females within child-bearing periods referred to the Gynecology Outpatient's Clinics of Fayoum and Al-Azhar University Hospitals in Egypt, who were complaining of vaginitis symptoms. When candida etiology was suspected, two upper vaginal swabs were obtained from each patient. Then the swabs were transported within 2 hours (hrs) to the Department of Medical Microbiology and Immunology, Faculty of Medicine, Fayoum University to be processed. The first swab was used to prepare a smear for Gram staining, and the second swab was inoculated on Sabouraud Dextrose Agar (SDA) (Oxoid Ltd., Hampshire, UK), with chloramphenicol, and incubated at 37 °C for 24-48 hrs. All strains were identified as to their species level by Viteck-2 System; according to the manufactures instructions. Pure *Candida* cultures were suspended in sterile glycerol broth vials and stored at -80 ºC, until required for further investigation.

Screening the isolates for voriconazole resistance

The susceptibility of the *Candida* isolates to voriconazole (1 µg, Oxoid) was performed using the Disk diffusion method; according to the Clinical and Laboratory Standards Institute guidelines[17].

Investigation of biofilm production

Candida biofilm production was assessed on pre-sterilized, polystyrene, flat bottomed, 96-well microtitre plates (Corning Inc., Corning, N.Y.): as described by Ramage et al[18]. Growth of biofilms were quantified using a crystal violet absorbance assay[19]. Each experiment was performed in triplicate. The optical density (OD) of each well was obtained using a microplate reader at 590 nm (Start –Fax spectrophotometer model 2100). The interpretation of the results was as follows: non biofilm producer (OD ≤ ODc), weak biofilm producer (ODc< OD ≤ 2 ODc), moderate biofilm producer (2 ODc< OD ≤ 4 ODc) and strong biofilm producer (4 ODc< OD); wherein: OD is the mean OD of each sample and ODc is the mean OD of the negative control[20].

Preparation of Kombucha tea culture

The tea fungus used in this study was of a traditional culture, provided by the Department of Agriculture Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The starter used was a symbiotic culture between yeast and acetic acid bacteria, mainly A. xylinum. Preparation of kombucha cultures from black tea was performed as described by Ismaiel et al[21]. Kombucha black tea was filtered and the fermented tea was then centrifuged for 10 min at 10,000 rpm, then the supernatant was separated and stored at 4-8 °C until required.

Determination of minimum inhibitory concentrations of Voriconazole with and without Kombucha

Minimum inhibitory concentration (MIC) of voriconazole against planktonic cells

Susceptibility testing of isolates to voriconazole was carried out by the micro-dilution method: as described by the Clinical and Laboratory Standards Institute[22]. Serial, two-fold dilution of voriconazole was performed in pre-sterilized, polystyrene rounded-bottomed, 96-well microtitre plates, with an RPMI 1640 medium (Sigma, St. Louis, MO);

concentrations ranged from 10 µg/ml to 0.0048 µg/ml. Each test strain had drug-free growth control wells, with media and *Candida* isolates. Another *Candida*-free well was included as a negative control well. The plates were incubated at 35 °C and read after 24 hours of incubation. Determination of endpoints was performed by visually comparing the growth in the wells containing the drug with that in the drug-free wells. Each experiment was performed in triplicate.

Minimum biofilm eradication concentration determination of voriconazole

Biofilms were formed on pre-sterilized, polystyrene, flat-bottom 96-well microtiter plates (Corning Inc., Corning, N.Y.): as described by Ramage et al[18]. After there was biofilm formation, the medium was aspirated, and non-adherent cells were removed by thoroughly washing the biofilms three times with sterile PBS. Residual PBS was removed by blotting with paper towels, before the addition of antifungal agents[23]. Voriconazole was then added to treat the pre-formed biofilms in serially two fold dilutions (from 10 to 0.0048µg/ml), from stock solutions of the antifungal agent prepared in RPMI medium and incubated for a further 48 hrs at 35 °C. A series of antifungal agent-free wells were included to serve as negative controls. Each experiment was performed in triplicate. The crystal violet absorbance assay was performed, and the minimum biofilm eradication concentrations ($MBEC_{80}$) were reported (80% eradication of pre-formed biofilms compared to control)[19].

Antifungal effect of Kombucha tea on minimum inhibitory concentration (MIC) of Voriconazole against Candida planktonic cells.

The broth microdilution method was used to determine the effect of Kombucha tea on the minimal inhibitory concentration (MIC) of voriconazole. This test was performed according to Deghrigue et al.[24] as follows: serial two-fold dilution of voriconazole solution in the supernatant of Kombucha tea was performed (diluted by Kombucha tea supernatant instead of RPMI 1640 medium) in 96-well microtitre plates. This is followed by the addition of 10 ul of *Candida* strain (containing 5×10^6 cfu/ml) to each well with mixing. Microtitre plates were incubated at 35 ˚C for 24 hrs to determine the effect of Kombucha tea supernatant on the MIC of voriconazole.

Antifungal effect of Kombucha tea on minimum biofilm eradication concentration of voriconazol against Candida Sessile cells

Biofilms were assessed on pre-sterilized, polystyrene, flat-bottom 96-well microtitre plates. After biofilm formation, the medium and non-adherent cells were removed by washing the biofilms three times with sterile PBS. Serial two-fold dilution of voriconazol solution in supernatant of Kombucha tea was prepared and added to preformed biofilms and incubated for a further 48 hrs at 35 °C[24]. $MBEC_{80}$ were determined for each strain by using the crystal violet absorbance assay[19].

Statistical analysis

Data were expressed in terms of frequencies and percentages. Comparison between the study groups was conducted using Chi-square (χ^2) test. When the expected frequency was less than 5, an exact test was used instead: p-values less than 0.05 were considered significant. All statistical calculations were caculated using the computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA), version 15 for Microsoft Windows.

Results

This study included 150 females within their child-bearing period, and had been referred to the Gynecology Outpatient's Clinics of Fayoum and Al-Azhar University

Hospitals, having complained of vaginitis symptoms. Eighty-nine *Candida* strains were isolated in the study, and from these 60 were voriconazole resistant, via the Disc diffusion method: 60/89 (67.4%) of the isolates were voriconazole resistant per the Disc diffusion method. Identification of these, via Viteck-2 System, revealed that: 35/60 (58.3%) isolates were *C. albicans*, 21/60 (35.0%) were *C. glabrata*, 2/60 (3.3%) were *C. famata*, 1/60 (1.7%) was *C. tropicalis* and 1/60 (1.7%) was *C. kruesi*.

Regarding biofilm production by isolated *Candida* strains, this study's results revealed that out of 60 strains, 48 (80.0%) were biofilm producers. Of these 21 (35.0%) were mild, 20 (33.3%) were moderate, and 7 (11.7%) were strong biofilm producers. Table 1 summarizes the ability for biofilm production by different species.

Testing the MICs of voriconazole revealed that of the 60 resistant isolates, via the Disc diffusion method, 57 were resistant and 3 had dose-dependent suscepability. These results revealed that: Kombucha tea culture supernatant significantly decreases the MIC_{50} of voriconazole against tested strains from 5 µg/ml to 0.625 µg/ml (p-value>0.01), and the MIC_{90} from 10 µg/ml to 1.25 µg/ml (p-value>0.01) (Table 2).

By studying the ability of voriconazole with and without Kombucha tea culture supernatant to eradicate *Candida* biofilms, it was found that voriconazole at a concentration of 0.156 µg/ml succeeded to eradicate biofilms formed by 18 strains; after adding the Kombucha tea supernatant, versus zero strains when using voriconazole alone. Also, biofilms eradication could not be reached for 11 strains when voriconazole alone was tested at a concentration of 10 µg/ml, versus 3 strains after adding the Kombucha tea supernatant (Table 3).

Table 1 The ability for biofilm production by different species

***Candida* isolates**	**Biofilm production**				**p-value**
	Non producer	**Mild**	**Moderate**	**Strong**	
Total isolates (N=60)	12 (20.0%)	21(35.0%)	20 (33.3%)	7 (11.0%)	0.797
C. albicans (N=35)	8 (22.9%)	11(31.4%)	13 (37.1%)	3 (8.6%)	
C. glabrata (N=21)	3 (14.3%)	8 (38.1%)	6 (28.6%)	4 (19.0%)	
C. non albicans non-glabrata (N=4)	1 (25.0%)	2 (50.0%)	1 (25.0%)	0 (0.0%)	

N=number of *Candida* isolates

Table 2 Voriconazole minimal inhibitory concentration against *Candida* planktonic cells

Candida planktonic cells antifungals susceptibility	**Voriconazole**	**Voriconazole & Kombucha tea supernatant**	**p-value**
MIC_{50} (µg/ml)	5 µg/ml	0.625 µg/ml	0.0001*
MIC_{90} (µg/ml)	>10 µg/ml	1.25 µg/ml	

MIC_{50}=minimal inhibitory concentration, which inhibit growth of 50% of Candida strains, MIC_{90}=minimal inhibitory concentration, which inhibit growth of 90% of Candida strains; a p-value<0.05 is considered significant

Table 3 Minimum Biofilm Eradication Concentrations ($MBEC_{80}$) of Voriconazole, with and without Kombucha tea culture supernatant

Voriconazole concentrations (µg/ml)	Voriconazole $MBEC_{80}$				Voriconazole & Kombucha $MBEC_{80}$			
	Total number of tested strains (N=48) N (%)	*C. albicans* (N=27) N (%)	*C. glabrata* (N=18) N (%)	*C. non albicans non-glabrata* (N=3) N (%)	Total number of tested strains (N=48) N (%)	*C. albicans* (N=27) N (%)	*C. glabrata* (N=18) N (%)	*C. non albicans non-glabrata* (N=3) N (%)
0.156	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	18 (37.5)	8 (29.6)	9 (50.0)	1 (33.3)
0.312	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (8.3)	3 (11.1)	1 (5.5)	0 (0.0)
0.625	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	9 (18.8)	7 (26)	1 (5.5)	1 (33.3)
1.25	4 (8.3)	3 (11.1)	1 (5.5)	0 (0.0)	6 (12.5)	3 (11.1)	2 (11.1)	1 (33.3)
2.5	6 (12.5)	3 (11.1)	3 (16.7)	0 (0.0)	5 (10.4)	4 (14.8)	1 (5.5)	0 (0.0)
5	18 (37.5)	13 (48.1)	3 (16.7)	2 (66.7)	2 (4.2)	1 (3.7)	1 (5.5)	0 (0.0)
10	9 (18.8)	3 (11.1)	5 (27.8)	1 (33.3)	1 (2)	1 (3.7)	0 (0.0)	0 (0.0)
>10	11 (22.9)	5 (18.5)	6 (33.3)	0 (0.0)	3 (6.3)	0 (0.0)	3 (16.7)	0 (0.0)

Discussion

Vulvovaginal candidiasis is a female genital tract infection affecting millions of subjects every year, and currently, it is considered the second cause of female genital infections[3]. Antifungal resistance is considered a major health problem; especially if the patient has been treated previously with a member of the azole group.[25] In this present study 60/89 (67.4%) of the isolates were voriconazole resistant, 57 (64.0%) were resistant and 3 (3.4%) isolates had dose-dependent suscepability): 35 (58.3%) isolates were *C. albicans*, 21 (35.0%) were *C. glabrata*, 2 (3.3%) were *C. famata*, one (1.7%) was *C. tropicalis* and one (1.7%) was *C. kruesi*. These present results are comparable to those reported by Adesiji and colleagues[26], who screened 26 *Candida* species causing VVC for voriconazole resistance and reported that 6 (23.1%) of them had dose dependent susceptible, with 16 (61.5%) being resistant. On the other hand, these results are higher than that reported by Waikhom et al., who tested 54 *Candida* strains isolated from VVC, and found that 37% of *Candida* strains were voriconazole resistant[27]. High resistant rates could be explained by the cross-resistance reported for members of azole antifungals on *Candida* isolates[28]. In addition, isolates from recurrent VVC may be more resistant to azoles than those isolated from VVC[25].

Treatment of uncomplicated VVC, caused by *C. albicans*, includes many options; for example: short-course topical imidazoles or ciclopirox olamine, azole vaginal suppositories, creams and also oral azoles[29]. In contrast, complicated vaginal candidiasis, which is usually caused by non-*C. albicans* species, requires a prolonged course of therapy. This form usually has difficultly in achieving successful treatment results; especially *C. glabrata* and *C. krusei*, as they do not respond to a single dose of azoles due to high resistant rates[11].

Previous studies reported that Kombucha tea culture had antimicrobial activity against some strains of *Candida* and bacteria. The antifungal activity of Kombucha is due

to the high production of acetic acid in the beverage[15,16]. The un-dissociated particles of the acid product, presenting during fermentation, may be absorbed into the fungus, causing dissociation to take place in the cytoplasm, and suppressing the organisms' metabolism. Hence Kombucha could be an excellent candidate for fighting the emergence of new, resistant microorganisms[30]. In this context, this study investigated the antifungal activity of voriconazole, with and without Kombucha tea cultures, against clinical *Candida* strains isolated from vulvovaginal candidiasis. These present results revealed that Kombucha tea culture supernatant significantly decreased the MIC_{50} of voriconazole against tested strains from 5 µg/ml to 0.625 µg/ml (p-value<0.01) and the MIC_{90} from 10 µg/ml to 1.25 µg/ml (p-value<0.01). The marked reduction of the MICs of voriconazole in combination with Kombucha reflects its powerful antifungal activities, which can be used as a new candidate for VVC therapy.

A few studies have investigated the antifungal activities of Kombucha; however to our knowledge none of them have investigated the *Candida* species isolated from VVC: additionally, all these researches only included a limited number of strains. Battikh et al. reported that a fermented infusion of Kombucha black tea processed antifungal activities against *C. albicans, C. tropicalis, C. glabrata,* and *C. dubliniensis*[16].

Biofilm is defined as a collection of microorganisms that are embedded in the polysaccharide matrix, and are attached to each other and to surfaces[31]. Biofilm formation by *C. albicans* in VVC leads to local inflammation and histopathological changes in the vaginal epithelium. In addition, biofilm formation leads to high resistance to antifungals, which promotes the formation of antifungal-tolerant persister *Candida* cells[32]. Accordingly, an important challenge will then be faced during treatment. This highlights the urgent requirement for the development of new anti-biofilm strategies that focus on alternative compounds, which can be used alone or in association with conventional drugs[33]. Regarding biofilm production by isolated *Candida* strains, this study's results revealed that out of 60 strains; 48 (80.0%) were biofilm producers. When investigating the ability of voriconazole, with and without Kombucha tea culture supernatant to eradicate *Candida* biofilms, it was found that voriconazole at a concentration of 0.156 µg/ml succeeded to eradicate biofilms formed by 18 strains. This was after adding the Kombucha tea supernatant, versus zero strains when using voriconazole alone. In agreement with our results, El-Azizi et al. found voriconazole failed to eradicate C. albicans biofilm and the resistance progressed, resulting in maturation of the biofilm[34]. Additionally, Fernandes et al. reported that voriconazole was partially able to control *C. tropicalis* biofilm formation, but was completely unable to eradicate pre-formed biofilms[35].

This study did not find any study investigating the anti-biofilm activity of Kombucha against *Candida* biofilm; hence, these present results, suggesting the powerful effect of Kombucha, could be the base of future researches. Further large-scale studies should be undertaken to investigate the antimicrobial and anti-biofilm activities of Kombucha alone, and in combination with traditional antifungals against fungal isolates implicated in human diseases.

Conclusion

In conclusion, Kombucha is a promising candidate for antifungal therapy; especially, when combined with the current therapeutic antifungals. It could even be used as a safe and low-cost prophylactic agent to protect from VVC; especially, in risk groups such as immunosuppressed, diabetic and pregnant women.

Conflict of interest

The authors declare no conflicts of interest.

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