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The association of air pollutants (CO_2, MTBE) on *Candida albicans* and *Candida glabrata* drug resistance

Original Study

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Abstract

Introduction. Therapeutic methods are very important in the prevalence of opportunistic fungal infections, which are an important cause of human diseases. In this study, air pollution agents that are in direct contact with microorganisms, and the effects of carbon sources using CO_2 and MTBE on growth of fungi, and particularly the evaluation of changes in the expression of interfering genes in susceptibility and drug resistance in these fungi, were investigated.
Materials and Methods. CCollecting samples and isolating *Candida glabrata* and *Candida albicans* with phenotypic methods were accomplished. We then evaluated the minimum inhibitory concentration (MIC) with the M27A4 protocol of CLSI. We adjusted 20 strains of *C. albican*s and 10 strains of *C. glabrata* whose sensitivity was evaluated in the MIC test with 5% CO_2 and 5mg/ml methyl tert-butyl ether (MTBE) considered as air pollutants, and followed by re-evaluating MIC testing to separate azole-resistant strains. Interfering agents were also considered.
Results. Upregulation of some genes on the two mentioned yeasts had led to drug resistance in them; they were previously sensitive to both drugs. Correspondingly, 41% of *C. glabrata* samples in sputum showed sensitivity to these drugs. Upregulation of *ERG11* (71%) and *EPA1* (90%) were observed in resistant strains. Upregulation of genes associated with aspartate proteins and downregulation of *SAP3* genes were recognized in *C. glabrata* in sputum and a 15% downregulation of bronchoalveolar lavage (BAL) isolate and 50% upregulation of *SAP1* gene in *C. albican*s sensitive samples were observed and compared to fluconazole and itraconazole with the oral and joint sources. Remarkably, decreased *SAP2* expression in oral sources and a 60% increase in resistant strains in *C. albican*s were observed. The downregulation of *SAP3* expression showed in the joint samples. An increase in *HWP1* expression (30%) was noted in isolated and drug-sensitive samples at the sputum and BAL source. *CDR1* expression was increased in MTBE-affected species; however, it decreased in the vicinity of CT.
Conclusions. Air pollutants such as CO_2 and MTBE eventually caused drug resistance in *Candida*, which can be one of the causes of drug resistance in candidiasis infections.

Keywords

Candida • air pollutant • CO_2, MTBE • drug resistance • molecular investigation

1. Introduction

Air pollution has become a major environmental challenge facing humanity in this century. Many agents, such as environmental elements, play an important role in human life. Geographical conditions, temperature, humidity, and pollution also have a major effect on the health or illness of humans and are considered to be a global threat [1].

Allergic syndrome, hypersensitivity syndrome, inflammation of paranasal sinusitis, itching, respiratory infection, and many other diseases have resulted from these elements. In this way, pollution has an impact on eukaryotic and prokaryote cells, and on humans [2]. Correspondingly, *Candida* is a prevalent microorganism in the reproductive and gastrointestinal mucosa that can be isolated from the oral cavity. For this reason, most of the healthy population are susceptible to the most prevalent fungal infections such as candidiasis. *Candida* species generate infections ranging from superficial diseases as well as systemic infections, especially in immunocompromised patients [3]. More than 150 species of *Candida* have been identified, but few were known to cause infections in humans, including *C. albican*s, *C. krusei, C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, *C. dubliniensis*, *C. kefyr*, *C. guilliermondii,* and *C. stellatoidea* [4]. Among all of these, *C. albican*s is the most pathogenic; *C. glabrata* is considered to have the highest frequency of drug resistance among all *non-C. albicans* species [5]. After *C. albicans, C. glabrata* is the most prevalent agent of mucosal and disseminated candidiasis in adults [6]. *C. albican*s generate extremely organized biofilms

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consisting of various cells, such as round, budding yeast-form cells; oval pseudohyphal-cells; long, cylindrical hyphal-cells enclosed in an extracellular matrix [7]. Presently, biofilm infection happens in more than 50% of these catheters even with newly enhanced clinical procedures, and these infections can cause serious health and financial implications. As fungal biofilms are mainly resistant to currently used antifungal drugs, the treatment of diseases usually takes higher doses of antifungals with removal of colonized medical devices [8]. In recent years, extensive studies have been performed on many virulence factors in *C. albicans*, including hyphal formation, phenotypic switching, and extracellular hydrolytic enzyme production. Hydrolytic enzyme production, which is recognized as one of the key agents in yeast pathogenicity, is a factor contributing to the process of virulence. Among the different types of hydrolytic enzymes found in microorganisms, the most common ones related to virulence are proteinases [9]. Secreted aspartyl proteinases (SAP), phospholipase B enzyme, and lipases are the most important extracellular hydrolytic enzymes produced by *C. albicans* [10]. The expression and regulation of 10 SAP genes increases the number of questions regarding the role and impact of these proteinases throughout the infection procedure. Temporary activation of 10 SAP genes throughout the various infection phases provides that parts of this gene family have an important function in *C. albicans*' response to the surroundings, especially its host [11]. Stresses and environmental agents, geographical condition, temperature, humidity, and pollution are associated with the presence of CO_2; MTBE is present due to unusual use of synthetic agents such as fuel, paint or polish. Many other environmental factors such as CO_2 and O_2 concentrations in high doses, PH, and other parameters can contribute by changes in normal growth, virulence factors, hydrophobicity, biofilm formation, secretory enzymes, and alteration of fungal drug resistance and other factors that relate to microorganisms [12].

Nowadays air pollution has affected many organisms such as fungi, and has created many changes in their features. One of these pollutants is MTBE (methyl tert-butyl ether), used as a fuel additive to improve and reduce greenhouse gases and other hazardous pollutants, used instead of lead in gasoline all around the world. This substance has a very high solubility that has a high risk for contamination of drinking water [13]. The addition of MTBE improves the physical characteristic of liquid fuels (used at 15% by volume). MTBE has aqueous solubility (>5 g L^{-1}), low sorption to soil and sediments, and volatile. Some microbial genera can use the compound and metabolize MTBE completely to CO_2; it is used as a carbon source by some prokaryotic organisms [14]. To find out the relation of CO_2 and MTBE exposure and drug resistance identification, the effects of CO_2 and methyl tertiary butyl ether ($C_5H_{12}O$-MTBE) concentration on *C. albicans* and *C. glabrata* growth and their virulence factors, this project was accomplished.

2. Materials and Methods

2.1. *Candida* isolation

In this study, clinical isolates obtained from two hospitals (Imam Khomeini and Shariati Hospitals, Tehran, Iran) were used. Previously isolated samples maintained in mycology collection in the school of public health at Tehran University of Medical Sciences, and standard strains of *C. albicans* (ATCC10231) and *C. glabrata* (ATCC90030) were involved as well.

About 105 patients with cutaneous, mucous, and deep infections of candidiasis were obtained in 2016-2017 from the mentioned centers. The samples included: nails, sputum, stool, BAL, groin, skin, and mouth. We investigated 33 samples of *C. albicans* and 17 samples of *C. glabrata* which were obtained from different locations in the patient's body. All samples were then cultured on SDA (sabouraud dextrose agar, Sigma) and incubated at 35 °C for 48 hours.

2.2. Morphological identification

All obtained yeasts were detected by morphological and molecular trends. For morphological identification, we used CHROMagar *Candida* medium (Paris, France) to identify *C. albicans* from multiple other *Candida* species based on the colors produced after 48 h of incubation. All yeast samples that were identified as *non-albicans* on the CHROMagar *Candida* medium have been forwarded for molecular identification by RFLP-PCR.

Besides, RFLP-PCR was utilized for verifying the identification of *C. albicans* and *C. glabrata* species used in this study.

2.3. Molecular identification

2.3.1. DNA extraction

For DNA extraction, 10^3 cells/ml of all isolates from fresh colonies were harvested, then the glass bead disruption method was done. Briefly, cultured yeasts were dissolved in 1.5 ml micro-centrifuge tube and 300 mg of 0.5 mm diameter glass beads, 300 µl of lysis buffer (100mM Tris-HCl pH 8, 10 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulphate), and 300 µl of phenol chloroform-isoamyl alcohol (25:24:1) were added. Then all samples were shaken for 5 min, centrifuged for 5 min at 5000 rpm. The supernatant was transferred to a fresh tube and extracted again with chloroform. High molecular weight DNA was precipitated by adding the same volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2). After that, the solution was completely vortexed and incubated for 10 min at – 20° C and centrifuged for 15 min at 12000 rpm. The precipitant was washed with ice-cold 70% ethanol, dried in the air, dissolved in 50 µl of double distilled water, and stored at -20° C until used for complementary identification of isolates.

Then for detection of *Candida* species, the RFLP method was applied by amplification of ITS1-5.8S-ITS2 of fungal rRNA genes fragment using ITS1, ITS4 universal primers (ITS1: 5′ TCCGTAGGT-

GAACCTGCGC 3', and ITS4: 5' TCCTGGGCTTATTGATATGC 3') and the Msp1 restriction enzyme (Fermentas, Germany). Briefly, 2.5 µl of 10× PCR buffer with $MgCl_2$, 0.4 mM of dNTP mix, 1 µl of Taq polymerase, 2 µl of DNA template were used for PCR. The PCR amplification was performed in Veriti 96 Thermal Cycler (Applied Biosystems, USA) based on the program: initial denaturation at 94° C for 3 min then 40 cycles at 94° C for 20 s, 55° C for 30 s and 72° C for 45 s, and subsequently final extension at 72° C for 5 min. The PCR amplicons were resolved with DNA markers in 2% agarose with ethidium bromide (0.5 µg/ml) by gel electrophoresis and for definition, *Candida* isolates. The PCR product was digested by Msp1 enzyme. Obtained *C. albicans* and *C. glabrata* species were selected to continue the procedure.

2.3.2. MIC (minimum inhibitory concentration) of two azole drugs

This test was performed based on broth microdilution Clinical and Laboratory Standard Institute (M27A4) method of CLSI with RPMI1640 (Gibco, USA). Briefly, *Candida* species were harvested from 24-48 hours of SDA culture and diluted with sterile phosphate buffer saline (PBS) to prepare 1- 5×10^3 CFU/ml by spectrophotometer (530 nm). MIC tests were then conducted based on CLSI protocol for fluconazole and itraconazole antifungals (Janssen Research Foundation, Beerse, Belgium). Drugs were used as reagent-grade powders dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) buffered to pH 7.0 with 0.165 mol · L^{-1} morpholine propane sulfonic acid buffer with L-glutamine without bicarbonate (MOPS, Sigma-Aldrich, St. Louis, MO, USA).

Only sensitive isolates were selected for being confronted with interference agents. We have had the same condition of CLSI protocol for all samples before and after they were confronted with the interference agents CO_2 and MTBE.

2.4. Interference agents

2.4.1. CO_2

CO_2 atmosphere was provided by using a 20-L cell culture CO_2 incubator (SLS, USA). The amount of CO_2 injection was monitored by the calibrated automatic control panel in this incubator.

In this study, we cultured all samples (30 samples) on SDA medium and incubated them at 37° C in a 5% CO_2 incubator for 1-4 weeks alternatively. Simultaneously, the samples were also cultured in the same condition without CO_2 for comparing the obtained morphological results.

2.4.2. MTBE

In this study, all samples were cultured on SDB containing 5 mg/ml MTBE and incubated at 37 degrees for 1-4 weeks alternatively.

2.5. Determination of MIC after treatment by CO_2 and MTBE

Alternatively, isolates were confronted with CO_2 and MTBE for four weeks. MIC tests were repeated to detect possible resistances to fluconazole and itraconazole. The same condition described previously was followed for MIC and all tests were performed in duplicate.

2.6. RNA extraction

The RNA molecules were extracted by the RNX-plus kit (Sinaclon Co., Iran) from all *Candida* species which gained resistance after being confronted with CO_2 and MTBE. Briefly, 10^3 cells/ml from fresh colonies were prepared and treated with the isolation reagent. The quantity and quality of the extracted RNA were then confirmed by absorption measurement at 260 and 280 nm using the spectrophotometer (Beckman Coulter, CA, USA) and electrophoresis on 1% agarose gel. The extracted RNA was stored at -80° C and used for cDNA synthesis with easy cDNA reverse transcription kit (Sinaclon Co., Iran) according to the instructions.

2.7. Real-time PCR after effect of agents

The obtained cDNAs were then utilized in real-time PCR assay with specific primers and by using AMPLICQON (Real Q plus 2x master mixes Green High Rox) in the ABI one step (Biosystems, Rotkreus, Switzerland) instrument. The mixture contained 10

Table 1. Sequences of primers used in Real-time PCR reaction in C. albicans and C. glabrata

Gene	Primer	Sequence (5'->3')
Sap1	Forward	TGGGTTCCTGATGCTTCTGTT
	Reverse	TCGGCAAAGACTTGCTTTGTG
Sap2	Forward	GGGGACATATGATCCAAGTGGT
	Reverse	CCACCGGCTTCATTGGTTTT
Sap3	Forward	ATGTTACTGGTCCCCAAGGTG
	Reverse	CCTTGACCAGCTTGACATGAA
HWP1	Forward	AATCATCAGCTCCTGCCACTG
	Reverse	GTCGTAGAGACGACAGCACTA
CDR1	Forward	GGTGCTAATATCCAATGTTGG
	Reverse	GTAATGGTTCTCTTTCAGCTG
EPA1	Forward	GGTCACTTACCCGCAAGCTA
	Reverse	CCAGATGGCGTAGGCTTGAT
ERG11	Forward	GAGATTGCACCACCCATTGC
	Reverse	TGGAGATAGCACCGAAACCG
β-actin	Forward	ACGGTATTGTTTCCAACTGGGACG
	Reverse	TGGAGCTTCGGTCAACAAAACTGG

Table 2. Dispersion the sources of Candida isolates

Nature of specimen	*C. albicans*	*C. glabrata*	Other spices	Number of *Candida* spp	%
BAL	8	3	8	19	18.02
SPUTUM	13	10	31	54	51.42
NAIL	6	2	4	12	11.42
MOUTH	2	-	2	4	3.8
GROIN	3	-	4	7	6.68
SKIN	1	1	4	6	5.81
STOOL	-	1	2	3	2.85
Total	33	17	60	105	

Table 3. Isolates of C. albicans and C. glabrata which were sensitive for both drugs with PCR-RFLP

TMML no	Source sample	MIC (μg/ml) Itr	MIC (μg/ml) Flu	*Candida* spp
TMML1	BAL	0.016	0.125	*albicans*
TMML2	sputum	0.25	2	*albicans*
TMML3	sputum	0.125	1	*albicans*
TMML4	sputum	0.5	1	*albicans*
TMML5	mouth	0.016	1	*albicans*
TMML6	nail	0.5	1	*albicans*
TMML7	nail	1	2	*albicans*
TMML8	BAL	0.016	1	*albicans*
TMML9	nail	0.062	0.5	*albicans*
TMML10	groin	0.016	0.5	*albicans*
TMML11	BAL	0.016	0.5	*albicans*
TMML12	BAL	0.062	0.5	*albicans*
TMML13	sputum	0.125	0.25	*albicans*
TMML14	sputum	0.125	0.25	*albicans*
TMML15	sputum	0.016	0.5	*albicans*
TMML16	sputum	0.016	0.25	*albicans*
TMML17	skin	0.5	2	*albicans*
TMML18	sputum	0.125	0.5	*albicans*
TMML19	groin	0.016	1	*albicans*
TMML20	sputum	0.031	0.5	*albicans*
TMML21	stool	1	8	*glabrata*
TMML22	sputum	0.5	8	*glabrata*
TMML23	sputum	1	16	*glabrata*
TMML24	sputum	1	8	*glabrata*
TMML25	sputum	0.25	8	*glabrata*
TMML26	sputum	1	16	*glabrata*
TMML27	sputum	0.125	16	*glabrata*
TMML28	sputum	1	8	*glabrata*
TMML29	skin	1	8	*glabrata*
TMML30	BAL	0.062	1	*glabrata*

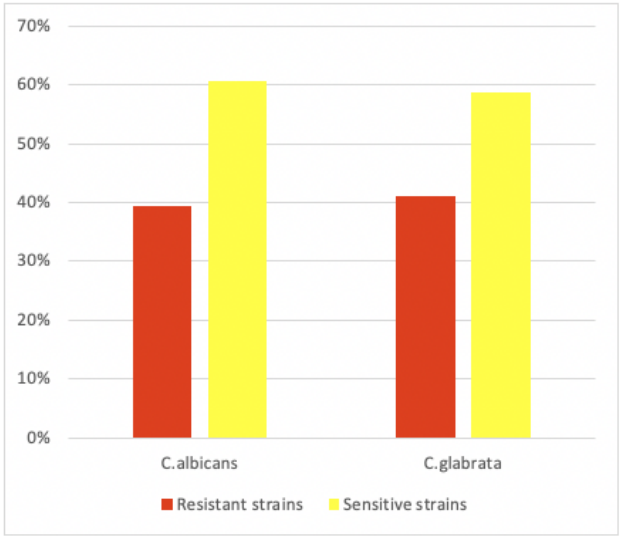


Fig. 1. Sensitivity pattern of C. albicans and C. glabrata

µl of master mix (Green High Rox), 1 µl of each specific primer (ERG11, CDR1, HWP1, EPA1, SAP1-3), and 2 µl of each cDNA sample. The mixture was adjusted to the final volume of 20 µl applying DEPC water. The program of real-time PCR was as follows: initial denaturation at 95° C for 2 minutes and followed by 40 cycles including in 95° C for 20 seconds, 59-60° C for 20 seconds, and 72° C for 30 seconds.

2.8. Primers

The specific primers (ERG11, CDR1, HWP1, EPA1, and SAP1-3) were designed by applying all-ID design software (Table 1) [15, 16, 17, 18, 19, 20].

The β-actin gene was used for the optimization of the real-time PCR as a housekeeping gene. The reactions were performed in duplicate and the analysis was done by REST2009 software.

3. Results

The effect of MTBE and CO_2 on drug sensitivity and some virulence factors in *C. albicans* and *C. glabrata* were assessed. *C. albicans* and *C. glabrata* isolates were then recognized based on the result of morphological and molecular tests. The CHROM agar candida medium was applied for morphological diagnosis and *C. albicans* with green color and *C. glabrata* with pink color were selected. Performed molecular complementary identification based on RFLP-PCR revealed all 105 isolated as *C. albicans* and *C. glabrata*. The total of 105 *Candida* species which were isolated from different sources of candidiasis involved patients in the mentioned hospitals, which are shown in Table 2.

The minimum inhibitory concentrations (MICs) against fluconazole and itraconazole were evaluated based on the M27-A4 method. Based on the obtained results, 20 samples of *C. albicans* and 10 samples of *C. glabrata* which were completely sensitive to both mentioned antifungals, were selected for continuing our research (Table 3, Figure 1). *C. albicans* selected sensitive species were isolated from BAL, sputum, mouth, skin, joint, and nail samples. *C. glabrata* selected sensitive species were isolated from BAL, stool, skin, and sputum samples. All these isolates were sensitive to both itraconazole and fluconazole. All 30 samples were cultured on SDA and SDB mediums simultaneously and were confronted with 5% CO_2 and 5mg/ml MTBE respectively. The cultured media were then incubated at 37° C for up to 4 weeks and were tested for MIC after two and four weeks of incubation. The initial changes were observed after 2-4 weeks of confronting with mentioned agents, and the resistant isolates against fluconazole and itraconazole were then recognized. Regarding *C. albicans*, the species that grew in ≥8 µg/ml of fluconazole was mentioned as resistant. However, this concentration has been mentioned as ≥ 64 µg/ml for *C. glabrata*. Furthermore, the MIC criteria for being mentioned as resistant against itraconazole was ≥1 µg/ml in both *C. albicans* and *C. glabrata* (Table 4).

The resistant species of both *C. albicans* and *C. glabrata* were then tested for possible changes in gene regulation by RT-PCR. The results indicated the upregulation of biofilm-associated genes in 57.1% of the sensitive *C. glabrata* species which isolated from sputum and became resistant against both antifungals.

In this study, most species increased the expression of genes associated with biofilm formation. 58.8% of all *C. glabrata* samples were sputum samples. Among the sputum samples, 42.8% were sensitive to both drugs, while 57.1% of the sensitive sputum samples became resistant to itraconazole after exposure to only interfering agents. Furthermore, an increase in expression of the EPA1 gene has been observed in about 70% of such sensitive samples. Regarding *ERG11* gene, upregulation and downregulation were observed respectively in 71% and 20% of resistant *C. glabrata* isolates. Increasing the gene expression in aspartyl protease gene and decrease of gene expression in the *SAP3* gene were observed in two groups, each consisting of 30% of *C. glabrata* isolates (Table 5, Figure 2).

In *C. albicans*, in 30% of the resistant species against both drugs, an upregulation in the *HWP1* gene was observed. Different changes in the regulation of other genes, which may be due to exposure to different interfering factors, were observed and revealed (Table 6, Figure 3).

The result of biofilm formation for *C. albicans* and *C. glabrata* according to the effects of CO_2 and MTBE is shown in Figures 4 and 5.

4. Discussion

Candida species are considered the most important fungal yeast pathogen in immunosuppressed patients and other patients who use broad-spectrum antibiotics more widely. Candidiasis infections have increased in the recent decade and the causa-

Table 4. MIC result after CO_2 exposure and MTBE in C. albicans and C. glabrata

No	Spp	After 2 weeks with %5 CO_2		After 4 weeks with %5 CO_2		After 2 weeks with $5_{mg/dl}$ MTBE		After 4 weeks with $5_{mg/dl}$ MTBE	
		Flu	Itra	Flu	Itra	Flu	Itra	Flu	Itra
TMML1	*C. glabrata*	4	1	4	1	2	2	32	8
TMML2	*C. glabrata*	2	0.5	2	0.5	1	0.5	16	2
TMML3	*C. glabrata*	2	0.5	4	0.5	0.5	1	16	2
TMML4	*C. glabrata*	2	0.5	4	1	0.5	0.25	16	4
TMML5	*C. glabrata*	2	0.5	2	0.5	2	0.5	16	4
TMML6	*C. glabrata*	4	0.5	64	16	0.5	1	32	2
TMML7	*C. glabrata*	2	0.5	2	0.5	0.5	0.062	0.5	0.125
TMML8	*C. glabrata*	2	0.5	4	0.5	1	0.5	32	4
TMML9	*C. glabrata*	4	1	64	16	1	1	16	8
TMML10	*C. glabrata*	2	0.125	64	16	0.5	0.25	32	2
TMML11	*C. albicans*	0.125	0.062	0.5	0.125	1	0.4	16	4
TMML12	*C. albicans*	64	16	64	16	2	2	64	16
TMML13	*C. albicans*	64	16	64	16	0.125	0.125	0.125	0.25
TMML14	*C. albicans*	64	16	64	16	0.5	0.25	1	0.25
TMML15	*C. albicans*	64	16	64	16	1	0.25	16	8
TMML16	*C. albicans*	64	16	64	16	4	2	64	16
TMML17	*C. albicans*	64	16	64	16	2	2	64	16
TMML18	*C. albicans*	0.025	0.025	64	16	1	0.25	16	16
TMML19	*C. albicans*	64	16	64	16	1	0.25	2	0.5
TMML20	*C. albicans*	64	16	64	16	0.5	0.5	1	0.5
TMML21	*C. albicans*	64	16	64	16	0.5	0.5	1	0.5
TMML22	*C. albicans*	64	16	64	16	0.25	0.25	1	0.5
TMML23	*C. albicans*	64	16	64	16	2	0.125	64	16
TMML24	*C. albicans*	64	16	64	16	0.5	1	0.5	0.5
TMML25	*C. albicans*	1	0.5	2	0.5	0.25	0.125	2	0.5
TMML26	*C. albicans*	0.062	0.031	64	16	0.25	0.125	0.25	0.125
TMML27	*C. albicans*	1	0.25	2	1	0.25	0.5	1	0.5
TMML28	*C. albicans*	64	16	64	16	0.25	0.062	0.25	0.25
TMML29	*C. albicans*	64	16	64	16	0.5	1	16	4
TMML30	*C. albicans*	64	16	64	16	0.25	0.062	0.25	0.5

Table 5. Expression of ERG11, EPA1, SAP3 genes in comparison with β-actin in C. glabrata

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
ERG11	TRG	1.0	0.876	0.045 - 29.445	0.000 - 123.640	0.894	
EPA1	TRG	1.0	64.669	1.893 - 866.949	0.438 - 8,060.454	0.000	UP
SAP3	TRG	1.0	0.745	0.009 - 17.387	0.000 - 434.218	0.784	
B-act	REF	1.0	1.000				

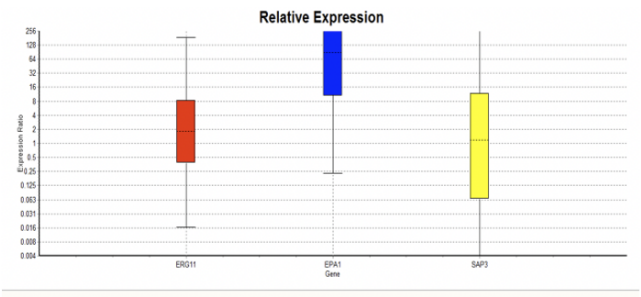


Fig. 2. Total result of C. glabrata. genes expression (ERG11, EPA1, SAP3)

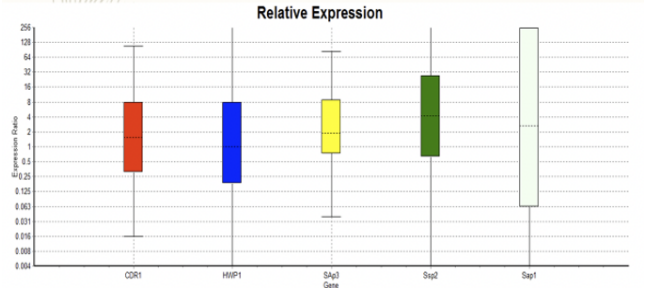
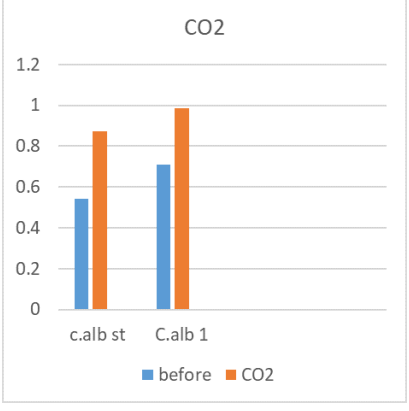


Fig. 3. Total result of C. albicans genes expression (CDR1, HWP1,SAP1-3)



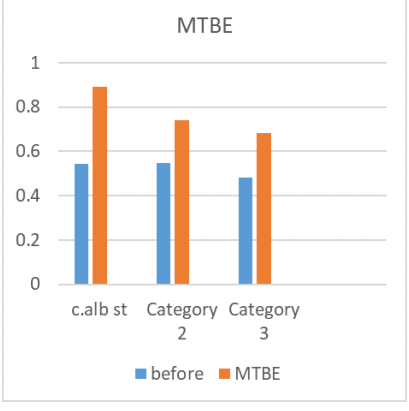
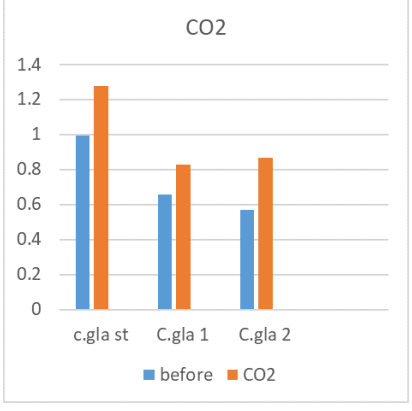


Fig. 4. Result of biofilm formation for C. albicans according to the effects of CO2 and MTBE



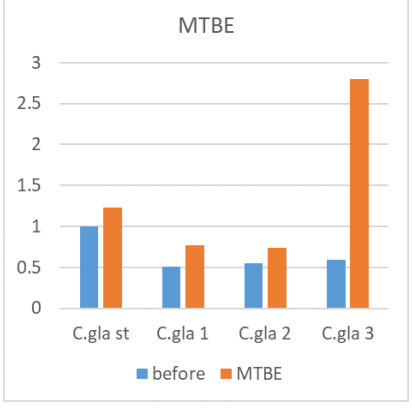


Fig. 5. Result of biofilm formation for C. glabrata according to the effects of CO2 and MTBE

tive agents may be affected by environmental changes such as CO_2 concentration, SN, MTBE, and other elements of air pollution [21]. Environmental changes including osmotic shock, carbon source, and oxygen concentration can affect the fungal cell wall. The cell walls in *C. albicans* consist of two layers: inner and outer. The outer layer is composed of the mannan fibrillary layer, and the inner layer contains β-glucan and chitin [22]. The vulnerability of the mannan layer emerged in response to increasing the temperature from 37° C in the early stage of biofilm formation. Mild heat stress can cause an increase in the thickness of the mycelial inner cell wall at 39° C [23]. *Candida* lysin secreted by *C. albicans* hyphae has an essential role in supporting the complicated structures of mature biofilms [24].

Air pollution such as MTBE and high concentrations of CO_2 (as an ubiquitous molecule) are among important environmental agents that threaten all living organisms, including yeasts. MTBE has been established as a human carcinogen by affecting oxidative stress and mitochondrial membrane and lysosomal membrane damages [13]. It has a negative effect on humans, such as a toxicity effect on human blood lymphocytes. This substance was used in liquid form with a purity of 99.9%. Evidently, the CO_2 concentration in humans is higher than air. The amount of CO_2 in the air is 0.03% and CO_2 concentration in human bodies is 4.5-30%. Therefore, the CO_2 content in humans is hundreds of times higher than in air. Recent research has reported that a low level of CO_2 can cause changes in some virulence factors in the gene level [25]. CO_2 is used for antimicrobial activity, especially the maintenance of foodstuffs, as it can impact microorganisms by inactivation of the cells.

However, in some countries, using MTBE is banned due to frequent soil and groundwater pollution by accidental spills from distribution systems and storage facilities. Butyl ether methyl is a gas additive that is added to increase the octane number and produced from methanol and iso-butylene. It is mainly used as a fuel oxygenating agent. The negative advantage of using this material is leak to surface and underwater minerals, but it is one of the major sources of contamination.

CO_2 could affect fungal physiology, morphology, and pathogenesis, respectively; it could also affect allergenic properties in pathogenic fungi. CO_2 concentration may also be considered an important pollution agent, affecting many features of yeast, which causes changes in its physiological and morphological characteristics. CO_2 is stimulus-inducing for filamentation in *C. albicans* and formation of pseudohyphae in *C. glabrata* yeasts is stimulated by CO_2 [26].

Kim and colleagues have indicated some changes occurred in *Cryptococcus neoformans* (synthase of polysaccharide capsule was increased) after exposure to 5% CO_2 atmosphere. Based on previous studies, 5% CO_2 atmosphere affected metabolism and pathogenesis of *C. neoformans* and *C. albicans*. In addition, some reports have shown that filamentation and pseudohyphal formation in *C. glabrata* is influenced under the effect of 5% CO_2 atmosphere. Synthase of *C. neoformans* polysaccharide capsule was increased after exposure to 5% CO_2 atmosphere. Also, changes were reported in *C. albicans* and *C. glabrata* which were drug-resistances due to exposure to 5% CO; expression of some virulence factors was also increased. Another study conducted by Yazdanparast and coworkers showed that certain conditions such as 10% CO_2 in *Trichophyton rubrum* can cause the formation of arthroconidia from hyphal cells, so in some, dermatophytosis with this agent may trigger antifungal resistance [27]. Some studies have considered the general inhibitory effect of CO_2 on fungal growth [28]. Some researches in 2004 announced that CO_2 pressure could have detrimental effects on growth and metabolism of yeasts, such as inactivation of *Saccharomyces cerevisiae* cells. CO_2 could change the C:N ratio and carbonic anhydrases (CAs) to affect the growth of fungal spores by protein production. Some reports indicated that some species of *C. glabrata* could produce pseudohyphal cells during nitrogen starvation. On other side, changes in CO_2 pressure and oxygen can affect cell activity of *S. cerevisiae* which leads to cell inactivation [29].

Recent investigation demonstrates that *C. glabrata* had an ability to respond to CO_2 pressure as a stimulus, and filamentation and pseudohyphal formation in *C. glabrata* under the effect of 5% CO_2 atmosphere was seen [26]. Shimoda et al. showed CO_2

Table 6. Expression of CDR1, HWP1, SAP1-3 genes in comparison with β-actin in C. albicans

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
CDR1	TRG	1.0	1.623	0.160 - 20.966	0.035 - 68.781	0.342	
HWP1	TRG	1.0	1.300	0.069 - 21.856	0.009 - 533.742	0.702	
SAp3	TRG	1.0	2.298	0.471 - 20.190	0.077 - 48.176	0.042	UP
Ssp2	TRG	1.0	4.547	0.388 - 86.223	0.007 - 849.223	0.024	UP
Sap1	TRG	1.0	2.243	0.025 - 763.031	0.000 - 12,429.932	0.491	
B-act	REF	1.0	1.000				

pressure and temperature are applied for antimicrobial activity, also used for the preservation of foodstuffs by inactivation of cells such as *Saccharomyces cerevisiae* cells [30].

Another study has shown that MTBE and other ethers were used as a carbon source during the growth of propane by some microorganisms such as *Mycobacterium* spp. [31]. Peter Roslev and coworkers demonstrated the lack of androgenic response and weak estrogenic response in *S. cerevisiae* by exposing to MTBE [2].

Several species of *Candida* have infectious symptoms caused by their virulence factors, such as cell adhesion, biofilm formation, white-opaque (w-o), switching, and morphological transition that cause true and pseudohyphal forms in *C. albicans*. The importance of this commensal fungal reaction is due to resistance against antifungal drugs. Also, some main reasons for drug resistance are morphological transition and biofilm formation [32]. Treatments are often not possible, and many patients are unable to tolerate taking higher doses of antifungal drugs due to possible damages to some organs, including the liver and kidney. The *Candida* biofilm resistance phenomenon was for the first time established and demonstrated in 1995 for *C. albicans* by Hawser and Douglas. In *Candida* species knowledge of contribution of extracellular DNA to biofilm matrix and overall structure is scarce [33]. *C. albicans* biofilm matrix is composed of carbohydrate, protein, phosphorus, and hexosamine. Biofilm of *C. glabrata* consists of multilayers of blastospores with high connections among them and a high level of carbohydrate and protein [34]. The higher density of cells may be related to the high resistance of *C. glabrata* biofilm to antifungal azoles and amphotericin B [35], although some parameters such as PH, temperature, and oxygen availability are considered as inductors of biofilm architecture alternation and antifungal sensibility [36]. In 2014, Fonseca demonstrated the high level of proteins and carbohydrates in the matrix extract from biofilms in *C. glabrata* treated with fluconazole [35]. *ERG11* is the most central point which increases the ergosterol production in *C. glabrata* cell membranes in response to azole. Probably during the early phase of biofilm growth efflux pumps in *C. albicans* and *C. glabrata* contribute to drug resistance [37].

Cell walls in *C. glabrata* have more mannoprotein that is linked to 1, 3β-glucan via 1,6-β glucan, and the largest group of mannoprotein is glycosylphosphatidylinositol (GPI). GPI-modified proteins are covalently bound to the wall by 1, 6-β glucan, which plays a key role in adhesion and biofilm formation. Cell wall protein and mannose/glucose ratio is higher in *C. glabrata* compared to *C. albicans* [6, 38]. Reducing drug resistance is considered the main goal of treatment for deep infection of candidiasis. In this study, we investigate the effect of a high concentration of CO_2 (5%) and gasoline exhaust gases such as MTBE on *C. albicans* and *C. glabrata* isolates recovered from the patients.

Molecular proteins are produced by the aspartyl proteinase gene which has an important role in the virulence of *Candida* species. SAP expression is strain- and source-dependent and plays a significant and dramatic role in infections caused by *Candida* spp. [39]. SAPs are encoded by at least ten distinct highly regulated genes in a multigene family (*SAP1 – SAP10*). According to previous studies some members of this family are considered specific for some species of *Candida*, for instance, *SAP 5-6* are exclusive to *C. albicans* but the lowest rate of proteinase production was found in *C. glabrata* and no specific SAP genes were detected in *C. glabrata* according to previous studies [40].

Recent studies indicated some pathogenic changes in *C. glabrata* and *C. albicans* in growth under CO_2 atmosphere; they also reported molecular changes by adenylyl cyclase Cyr1 that promote white-to-opaque switching and then affect mating changes in *C. albicans* [25, 26]. Our results indicate that MICs of itraconazole and fluconazole against *C. albicans* and *C. glabrata* increased after confronting with some 5% CO_2 pressure and 5mg/ml MTBE. However, the mechanism of resistance remained inconspicuous. We used these environmental factors to induce drug resistance in two species of *Candida*. The final MICs were relatively higher than the initial ones.

Shabir Ahmad Lone and coworkers in 2019 had claimed that the new antifungal eugenol tosylate congeners (ETC-5, ETC-6, and ETC-7) have a fungicidal effect on *Candida* spp. They have also shown the downregulation of the *ERG11* gene which is related to ergosterol synthesis [41].

In the present study, expression of secretory enzymes including aspartyl protease (SAP) and biofilm formation and study of drug sensitivity modification (MIC) are evaluated by real-time PCR. We evaluated the effect of CO_2 and MTBE on drug susceptibility and some virulence factors of *C. glabrata* and *C. albicans* isolates that were gained from clinical samples. The obtained results indicated that CO_2 atmosphere and MTBE as two air pollution elements could enhance some virulence factors such as *SAP1-3* in *C. glabrata* and *C. albicans*. These interferences could cause drug resistance in some species which were susceptible before confronted with CO_2 or MTBE. Most of the mentioned changes were observed after 2-4 weeks of incubation under a 5% CO_2 atmosphere or 5mg/ml MTBE.

In addition to molecular studies of the aforementioned genes, the function of other genes along with the epigenetic and genetic investigation of these genes in molecular pathways is important.

5. Conclusions

Some genes associated with *C. albicans* and *C. glabrata* virulence factors were increased by gaining resistance against antifungal drugs and due to confronting with air pollutants such as CO_2 and MTBE.

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Authors' Contribution
S.G.: first author, accomplished the data processing, investigated the informatics database and also performed the statistical analyses, operated all the tests and wrote the whole article as the PhD student; **S.R.M.:** accompanied in all sections of this project as supervisor professor and edited the revised the manuscript comprehensively; **S.R.:** accompanied in all sections of this project as supervisor professor and edited the revised the manuscript comprehensively; **S.K.:** as the advisor professor was involved in in this project and edited and revised the manuscript completely; **A.A.S.:** as the advisor professor was involved in in this project and edited and revised the manuscript completely; All authors revised the article completely and confirmed the final edited version of the paper.

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Conflict of Interest
The authors have no potential conflicts of interest to declare.

Ethics Approval
Due to the nature of the research, the consent of the ethics committee was not required.

References

[1] Wei K, Qiu M, Zhang R, Zhou L, Zhang T, Yao M, Luo C. Single Living yEast PM Toxicity Sensor (SLEPTor) system. J Aerosol Sci. 2017; 107: 65-73.
[2] Roslev P, Lentz T, Hesselsoe M. Microbial toxicity of methyl tert-butyl ether (MTBE) determined with fluorescent and luminescent bioassays. Chemosphere. 2015; 120: 284-291.
[3] Hani U, Shivakumar HG, Vaghela R, Osmani RA, Shrivastava A. Candidiasis: A fungal infection-current challenges and progress in prevention and treatment. Infect Disord Drug Targets. 2015; 15: 42-52.
[4] Krcmery V, Barnes AJ. Non-albicans Candida spp. causing fungaemia: Pathogenicity and antifungal resistance. J Hosp Infect. 2002; 50: 243-260.
[5] Singh A, Healey KR, Yadav P, Upadhyaya G, Sachdeva N, Sarma S, Kumar A, Tarai B, Perlin DS, Chowdhary A. Absence of azole or echinocandin resistance in Candida glabrata isolates in India despite background prevalence of strains with defects in the DNA mismatch repair pathway. Antimicrob Agents Chemother. 2018; 62: e00195-18.
[6] de Groot PW, Kraneveld EA, Yin QY, Dekker HL, Groß U, Crielaard W, de Koster CG, Bader O, Klis FM, Weig M. The cell wall of the human pathogen *Candida glabrata*: Differential incorporation of novel adhesin-like wall proteins. Eukaryot Cell. 2008; 7: 1951-1964.
[7] Fox EP, Nobile CJ. A sticky situation: Untangling the transcriptional network controlling biofilm development in *Candida albicans*. Transcription. 2012; 3: 315-322.
[8] Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, Meersseman W, Akova M, Arendrup MC, Arikan-Akdagli S, et al. ESCMID* guideline for the diagnosis and management of Candida diseases 2012: Non-neutropenic adult patients. Clin Microbiol Infect. 2012; 18: 19-37.
[9] Calderone RA, Fonzi WA. Virulence factors of *Candida albicans*. Trends Microbiol. 2001; 9: 327-335.
[10] Naglik J, Albrecht A, Bader O, Hube B. *Candida albicans* proteinases and host/pathogen interactions. Cel. Microbiol. 2004; 6: 915-926.
[11] Kadry AA, El-Ganiny AM, El-Baz AM. Relationship between Sap prevalence and biofilm formation among resistant clinical isolates of *Candida albicans*. Afr Health Sci. 2018; 18: 1166-1174.
[12] Alfonso-Gordillo G, Flores-Ortiz CM, Morales-Barrera L, Cristiani-Urbina E. Biodegradation of methyl tertiary butyl ether (MTBE) by a microbial consortium in a continuous up-flow packed-bed biofilm reactor: Kinetic study, metabolite identification and toxicity bioassays. PLoS One. 2016; 11: e0167494.
[13] Salimi A, Vaghar-Moussavi M, Seydi E, Pourahmad J. Toxicity of methyl tertiary-butyl ether on human blood lymphocytes. Environ Sci Pollut Res Int. 2016; 23: 8556-8564.
[14] Juwono H, Yamin A, Alfian R, Ni'mah YL, Harmami H. Production of liquid fuel from plastic waste with co-reactan nyamplung oil (callophyllum inophyllum) and its performance in gasoline machine by adding MTBE additive. AIP Conf Proc. 2018; 2049: 020081
[15] Hube B, Sanglard D, Odds FC, Hess D, Monod M, Schäfer W, Brown AJ, Gow NA. Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. Infect Immun. 1997; 65: 3529-3538.
[16] Modrzewska B, Kurnatowski P, Khalid K. Comparison of proteolytic activity of *Candida* sp. strains depending on their origin. J Mycol Med. 2016; 26: 138-147.
[17] de Barros PP, Freire F, Rossoni RD, Junqueira JC, Jorge AO. *Candida krusei* and *Candida glabrata* reduce the filamentation of *Candida albicans* by downregulating expression of HWP1 gene. Folia Microbiol. 2017; 62: 317-323.

[18] Feng W, Yang J, Wang Y, Chen J, Xi Z, Qiao Z. *ERG11* mutations and up-regulation in clinical itraconazole-resistant isolates of *Candida krusei*. Can J Microbiol. 2016; 62: 938-943.

[19] Gallegos-García V, Pan SJ, Juárez-Cepeda J, Ramírez-Zavaleta CY, Martin-del-Campo MB, Martínez-Jiménez V, Castaño I, Cormack B, De Las Peñas A. A novel downstream regulatory element cooperates with the silencing machinery to repress *EPA1* expression in *Candida glabrata*. Genetics. 2012; 190: 1285-1297.

[20] Zhu SL, Yan L, Zhang YX, Jiang ZH, Gao PH, Qiu Y, Wang L, Zhao MZ, Ni TJ, Cai Z, et al.: Berberine inhibits fluphenazine-induced up-regulation of *CDR1* in *Candida albicans*. Biol Pharm Bull. 2014; 37: 268-273.

[21] Tobal JM, da Silva Ferreina Balieiro ME. Role of carbonic anhydrases in pathogenic micro-organisms: A focus on *Aspergillus fumigatus*. J Med Microbiol. 2014; 63: 15-27.

[22] Levin DE. Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: The cell wall integrity signaling pathway. Genetics. 2011; 189: 1145-1175.

[23]Ikezaki S., Cho T, Nagao JI, Tasaki S, Yamaguchi M, Arita-Morioka KI, Yasumatsu K, Chibana H, Ikebe T, Tanaka Y. Mild heat stress affects on the cell wall structure in *Candida albicans* biofilm. Med Mycol J. 2019; 60: 29-37.

[24] Abu El-Asrar AM, Missotten L, Geboes K. Expression of hypoxia-inducible factor-1α and the protein products of its target genes in diabetic fibrovascular epiretinal membranes. Br J Ophthalmol. 2007; 91: 822-826.

[25] Du H, Guan G, Xie J, Cottier F, Sun Y, Jia W, Mühlschlegel FA, Huang G. The transcription factor Flo8 mediates CO_2 sensing in the human fungal pathogen *Candida albicans*. Mol Biol Cell. 2012; 23: 2692-2701.

[26] Sasani E, Khodavaisy S, Agha Kuchak Afshari S, Darabian S, Aala F, Rezaie S. Pseudohyphae formation in *Candida glabrata* due to CO_2 exposure. Curr Med Mycol. 2016; 2: 49-52.

[27] Yazdanparast SA, Barton RC. Arthroconidia production in *Trichophyton rubrum* and a new *ex vivo* model of onychomycosis. J Med Microbiol. 2006; 55: 1577-1581.

[28] Papagianni M. Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol Adv. 2004; 22: 189-259.

[29] Coelho M, Belo I, Pinheiro R, Amaral A, Mota M, Coutinho J, Ferreira E. Effect of hyperbaric stress on yeast morphology: Study by automated image analysis. Appl Microbiol Biotechnol. 2004; 66: 318-324.

[30] Shimoda M, Cocunubo-Castellanos J, Kago H, Miyake M, Osajima Y, Hayakawa I. The influence of dissolved CO_2 concentration on the death kinetics of *Saccharomyces cerevisiae*. J Appl Microbiol. 2001; 91: 306-311.

[31] Tupa PR, Masuda H. Genomic analysis of propane metabolism in methyl tert-butyl ether-degrading *Mycobacterium* sp. strain ENV421. J Genomics. 2018; 6: 24-29.

[32] Graybill JR. The long and the short of antifungal therapy. Infect Dis Clin North Am. 1988; 2: 805-825.

[33] Harvey RJ, Lund VJ. Biofilms and chronic rhinosinusitis: Systematic review of evidence, current concepts and directions for research. Rhinology. 2007; 45: 3-13.

[34] Silva S, Henriques M, Martins A, Oliveira R, Williams D, Azeredo J. Biofilms of non-*Candida albicans Candida* species: Quantification, structure and matrix composition. Med Mycol. 2009; 47: 681-689.

[35] Fonseca E, Silva S, Rodrigues CF, Alves CT, Azeredo J, Henriques M. Effects of fluconazole on *Candida glabrata* biofilms and its relationship with ABC transporter gene expression. Biofouling. 2014; 30: 447-457.

[36] Pettit RK, Repp KK, Hazen KC. Temperature affects the susceptibility of *Cryptococcus neoformans* biofilms to antifungal agents. Med Mycol J. 2010; 48: 421-426.

[37] Akins RA. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. Med Mycol J. 2005; 43: 285-318.

[38] Klis FM, De Groot P, Brul S. 13 identification, characterization, and phenotypic analysis of covalently linked cell wall proteins. Methods Microbiol. 2007; 36: 281-301.

[39] Newport G, Agabian N. KEX2 influences *Candida albicans* proteinase secretion and hyphal formation. J Biol Chem. 1997; 272: 28954-28961.

[40] Dabiri S, Shams-Ghahfarokhi M, Razzaghi-Abyaneh M. SAP (1-3) gene expression in high proteinase producer Candida species strains isolated from Iranian patients with different Candidosis. J Pure Appl Microbiol. 2016; 10: 1891-1896.

[41] Lone SA, Khan S, Ahmad A. Inhibition of ergosterol synthesis in Candida albicans by novel eugenol tosylate congeners targeting sterol 14α-demethylase (CYP51) enzyme. Arch Microbiol. 2020; 202: 711-726.