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Original article

Antifungal resistance profiles of *Candida* isolates from pediatric tertiary care hospital and in vitro efficacy of natural oils against fluconazole-resistant species

Heba Hassan Ahmed [1], Yousseria M Shetaia [1], Ashraf M Morgan [2], Marwa A Ibrahim [* 3], Noha S Soliman [4]

1- Microbiology (Mycology) Department, Faculty of Science, Ain-Shams University, Egypt.
2- Toxicology and Forensic Medicine Department, Faculty of Veterinary Medicine, Cairo University, Egypt.
3- Biochemistry and Molecular biology Department, Faculty of Veterinary Medicine, Cairo University, Egypt.
4- Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University, Egypt.

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ABSTRACT

Background: Candidiasis is considered the most significant fungal infection with increasingly reported antifungal resistance, especially against fluconazole. **Aim:** This study aimed to determine rates of antifungal resistance among *Candida* isolates from pediatric clinical samples, and test the *in-vitro* efficacy of natural essential oils against fluconazole-resistant isolates. **Methods:** Identification and antifungal susceptibility testing were performed utilizing the Vitek2 compact automated system. Fluconazole-resistant *Candida* isolates were subjected to *in-vitro* screening for the efficacy of nine natural oils by disc diffusion method, followed by determining the minimal inhibitory concentration for the most effective oils using broth micro dilution. **Results:** Out of total cultured samples (n=2120), *Candida* were isolated at a rate of 6.27% (n= 133), mostly from urine and blood samples. *Candida albicans* was the most prevalent isolated species (54.9%), followed by *Candida tropicalis* (33.83%). Fluconazole recorded a higher resistance rate (9%) than the rest of antifungals, with a significantly higher incidence among non-*C. albicans Candida* species (NCAC) than *C. albicans* (p <0.05). Cinnamon, cumin, thyme, and lemongrass showed the widest mean of inhibition zones, of which cinnamon oil had the lowest MIC values against *Candida* isolates. **Conclusions:** Our study concluded higher predominance of *C. albicans* over NCAC species, and total fluconazole resistance rate of 9%. cinnamon oil was found to be the most effective oil against fluconazole-resistant isolates.

Introduction

The incidence of fungal infections, particularly candidiasis in children and immunocompromised patients, has been increasingly reported. *Candida* spp. exist as normal flora and colonizers of the skin, oral cavity, gastrointestinal tract and vagina. Nevertheless, specific species act as opportunistic pathogens causing severe infections, such as blood stream candidemia, candiduria, and other local and

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* *Corresponding author:* Marwa Ibrahim
E-mail address: marwa199@gmail.com

systemic infections, resulting in high mortalities [1]. *Candida albicans* (*C. albicans)* was reported as the chief etiological species of *Candida* involved in human infections [2]. Recent years have witnessed a rise in non- *C. albicans Candida* species (NCAC) including C. *tropicalis, C. glabrata, C. parapsilosis* and *C. krusei. Candida* species vary in their antifungal susceptibility, where resistance has continuously developed to most antifungal drugs, particularly azoles [3]. Among azoles, fluconazole is the most frequently used antifungal drug, since it is inexpensive and effective [4]. Fluconazole-resistance rates are continuously increasing and have become a driving cause for therapeutic failure in *Candida* infections, resulting in adverse clinical outcomes [5].

The continuously evolving resistance of *Candida* species against the current antifungal drugs prompted researchers to investigate new effective biocides [6]. In combating pathogenic *Candida* species, essential oils can act against a variety of targets, such as cell membranes and cytoplasm, and can entirely alter cell morphology [7]. Several studies investigated the antifungal efficacy of different plant natural oils against *Candida*, although there is still paucity of information in this field [8]. In this perspective, the present work aimed to overview different types of *Candida* species causing infections among children and rates of antifungal resistance, in addition to testing the in-vitro efficacy of natural essential oils against fluconazole-resistant isolates.

Methods

The study was carried out on collected *Candida* isolates from clinical samples routinely delivered to the microbiology laboratory in a pediatric tertiary-care hospital. Data on the source and type of samples, patients' age and gender were obtained from electronic laboratory records. The present study was approved by the Research Ethics Committee (REC) of the Faculty of Science, Ain-Shams University and the institutional review board of El-Mounira Children Cairo University Hospital.

Isolation and identification of *Candida* species

All specimens were cultured on routine microbiological media according to the standards of microbiological procedures [9]. For the isolation of *Candida*, the samples were simultaneously cultured on Sabouraud dextrose agar supplemented with chloramphenicol and gentamicin (Bio-Rad, California). All cultured plates were incubated aerobically at 35±2°C for 24 to 48 hours and isolates were taken from culture plates with pure growth of *Candida* [1].

Candida isolates were primarily identified by their colony morphology and Gram-stained smears. The germ tube was used to classify *Candida* into *C. albicans* and NCAC species [2]. Species differentiation of *Candida* isolates was obtained by an automated Vitek®2 Compact system (BioMérieux, France) utilizing colorimetric reagent identification yeast cards (ID-YST card). The *Candida* suspension was optimized to 2-2.4 McFarland using the Vitek2 Densi-Check [10]. The *Candida* species with confidence level of identification below 95% by Vitek2 system were confirmed by using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) Vitek® MS V2.0 (Biomerieux, France) according to the manufacturer's instructions [11].

Antifungal susceptibility testing

The antifungal susceptibility of *Candida* isolates was performed using Vitek®2 Compact automated system's YS07 card (BioMérieux, France), which is predesigned to determine the minimum inhibitory concentration (MIC) for a panel of 6 antifungals (fluconazole, micafungin, voriconazole, caspofungin, flucytosine and amphotericin B). The MIC results for the antifungals were interpreted according to the Clinical and Laboratory Standard Institute document M60 [12]. For amphotericin B, the MIC was interpreted based on the guidelines of the European Committee of Antimicrobial susceptibility testing [13]. For flucytosine, interpretive MIC breakpoints were used as follows: susceptible: ≥4 µg /ml), intermediate: 8–16 µg/ml, and resistant: ≥32 µg/ml) [14]. The *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019 strains were used to assure the quality of performance [10, 12, 15].

In vitro efficacy of natural oils against fluconazole-resistant *Candida* isolates

Nine types of crude oils (100% V/V) extracted from the plants in the form of Cumin seeds (Cuminum cyminum), clove (Eugenia caryophyllus), rosemary (Rosmarinus officinalis L.), cinnamon (Cinnamomum), peppermint (Mentha piperita), lemongrass (Cymbopogon citratus), anise (Pimpinella anisum), castor (Ricinus Communis) and thyme (Thymus vulgaris) were screened for their antifungal efficacy against fluconazole-

resistant *Candida* isolates using standardized disk diffusion method [16]. Sabouraud dextrose agar (SDA) was inoculated by *Candida* suspension at a concentration of $5x10^6$ CFU/ml. Plant oils (5µl) were spotted onto 6 mm discs and applied to the inoculated plates then, incubated at 35±2°C.The four most effective oils with the widest inhibitory zones were selected to be tested through two-fold concentrations (0.03% to 2% v/v) using the broth micro dilution (BMD) method for the determination of the MIC [8]. These concentrations were prepared by doubling dilution, and tween 80 was included to enhance oil solubility at a concentration of 0.001% (v/v). In a 96-wells plate, 100 µl was taken from each oil concentration and added to 100 µl of *Candida* inoculum, then incubated for 48 hours [8].

Statistical analysis

Data were described in the form of mean, standard deviation (SD), range, or frequencies, percentages and Chi-square (x^2). Statistical significance was set at P-values less than 0.05. All statistical calculations were done using the SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 16 for Microsoft Windows.

Results

Among the total number of cultured samples (n=2120) delivered to the microbiology laboratory, 133 demonstrated positive growth of *Candida* spp. (6.27%) that were isolated from 88 males (66.2%) and 45 females (33.8%). The majority of the isolated *Candida* spp. was significantly detected in the age group of ≤ 1 year (44.4%) compared to other age groups (*p*: 0.026), with the species distributed as shown in **figure (1).**

The frequency of isolation of different *Candida* species is illustrated in **figure (2),** where *C. albicans* had a higher frequency of isolation (n=73, 54.9%), than the NCAC (n=60, 45.1%). *Candida tropicalis* and *C. parapsilosis* were the most frequently isolated NCAC species at rates of 33.8% and 5.3%, respectively.

The distribution of *Candida* species among different types of samples had a statistically significant difference (*p*-value= 0.018). Urine samples had the highest proportion of *Candida* isolation (90/133, 67.6%), followed by blood samples (29/133, 21.8%) among all positive *Candida* cultures **(Table 1).**

Predominant isolation of *C. albicans* over NCAC species was observed in all types of samples except for endotracheal aspirate (ETA) and peritoneal fluid samples, which demonstrated equal recovery of *C. albicans* and *C. tropicalis*. In blood samples, *C. albicans* was the most prevalent species in blood samples (n=22, 76%), followed by *C. parapsilosis* (n=4, 14%) and *C. tropicalis* (n=2, 7%) as NCAC species. In urine and ETA samples, *C. tropicalis* was the predominant non-albicans species, with rates of isolation of 42.2% and 37.5%, respectively. Each of *C. krusei* and C. utilis was isolated twice from urine samples. Single recovery of each of *C. glabrata*, *C. lusitaniae*, and C. *pelliculosa* occurred from urine, ETA, and blood samples, respectively.

Candida species were isolated from patients in wards and ICUs at rates of 42% and 37%, respectively, showing predominance of *C. albicans* over NCAC species with isolation rates of 53.5% and 46.4%, respectively. Among outpatients, *Candida* had an isolation rate of 18%, where NCAC species were more frequently encountered than *C. albicans* at a rate of 66.6%, with the highest proportion for *C. tropicalis*. A statistically significant difference was observed in the distribution of *Candida* species among different hospital locations **(Table 2).**

Susceptibility of *Candida* species to antifungal drugs

Fluconazole exhibited the highest rate of antifungal resistance (n=12, 9%) among all *Candida* isolates (n=133). Fluconazole resistance was more encountered among NCAC species (n=8, 67%) than *C. albicans* (n=4, 33%), including the intrinsically resistant *C. krusei*. Only four NCAC species (2 *C. tropicalis* and *2 C. krusei*) were resistant to flucytosine, while one *C. tropicalis* isolate was resistant to voriconazole and caspofungin **(Table 3).** No resistance was detected with micafungin or amphotericin B. There was a statistically significant difference in fluconazole and flucytosine resistance rates among *Candida* species **(Table 3).**

Disk diffusion inhibitory zones and broth micro dilution MIC results of essential oils against fluconazole-resistant *Candida* isolates

The screening results of the antifungal efficacy of the nine natural oils against fluconazole-resistant *Candida* isolates showed that cinnamon, cumin, thyme, and lemongrass had the widest mean of inhibition zones, thus were selected to determine their MICs (% v/v) against fluconazole-resistant *Candida* isolates using the broth micro-dilution method **(Table 4).**

Cinnamon oil exhibited the lowest mean of MIC values (0.06-0.09% v/v), while cumin showed the highest mean of MIC values (>2% v/v) against all fluconazole-resistant *Candida* isolates tested. Lemon-grass and thyme exhibited comparable MICs (0.12% v/v) against all fluconazole-resistant *Candida* isolates tested, with the exception of *C. Krusei*, which exhibited a lower mean of MICs (0.06 % v/v) with lemongrass **(Table 5).**

Table 1. The Distribution of *Candida* species among different clinical samples of (n=133).

	***Candida* species No. (%)**								
Sample type	***C. albicans***	***C. tropicalis***	***C. parapsilosis***	***C. utilis***	***C. krusei***	***C. glabrata***	***C. lustaniae***	***C. pelliculosa***	**Total**
Blood	22	2	4	0	0	0	0	1	29
	(75.9)	(6.9)	(13.8)	(0)	(0)	(0)	(0)	(3.4)	(100)
endotracheal aspiration (ETA)	3	3	0	1	0	0	1	0	8
	(37.5)	(37.5)	(0)	(12.5)	(0)	(0)	(12.5)	(0)	(100)
peritoneal fluid	1	1	0	0	0	0	0	0	2
	(50)	(50)	(0)	(0)	(0)	(0)	(0)	(0)	(100)
wound swab	2	1	1	0	0	0	0	0	4
	(50)	(25)	(25)	(0)	(0)	(0)	(0)	(0)	(100)
urine	45	38	2	2	2	1	0	0	90
	(50)	(42.2)	(2.2)	(2.2)	(2.2)	(1.1)	(0)	(0)	(100)
Total	73	45	7	3	2	1	1	1	133
	(54.9)	(33.8)	(5.3)	(2.3)	(1.5)	(0.8)	(0.8)	(0.8)	(100)
Pearson Chi square (X^2)	45.8								
***P*-value**	0.018								

Table 2. Incidence of the isolated *Candida* species among different sources of clinical samples.

Source		*C. albicans* N (%)	*C. tropicalis* N (%)	*C. parapsilosis* N (%)	*C. krusei* N (%)	*C. utilis* N (%)	*C. glabrata* N (%)	*C. lustaniae* N (%)	*C. pelliculosa* N (%)	Total
In-patients	**NICU**	33 (45)	9 (90)	5 (71)	0	0	0	1 (100)	1 (100)	49 (37)
	Ward	30 (41)	22 (49)	2 (29)	0	1 (33)	1 (100)	0	0	56 (42)
Out-patients		8 (11)	13 (29)	0	1 (50)	2 (67)	0	0	0	24 (18)
Kidney transplantation unit		2 (3)	1 (2)	0	1 (50)	0	0	0	0	4 (3)
Total		73 (54.9)	45 (33.9)	7 (5.2)	2 (1.5)	3 (2.25)	1 (0.75)	1 (0.75)	1 (0.75)	133 (100)
Pearson Chi-square (X^2)		41.864								
***P*-value**		0.004								

NICU: Neonates intensive care units

Table 3. Antifungal susceptibility profile of different *Candida* species

Type of isolates	Fluconazole			Voriconazole			Caspofungin			Micafungin			Amphotericin b			Flucytocine		
	S N (%)	SDD N (%)	R N (%)	S N (%)	I N (%)	R N (%)	S N (%)	I N (%)	R N (%)	S N (%)	I N (%)	R N (%)	S N (%)	I N (%)	R N (%)	S N (%)	I N (%)	R N (%)
***C. albicans* (CA) (n=73)**	69 (58)	0 (0)	4 (33)	73 (55)	0 (0)	0 (0)	73 (55.7)	0 (0)	0 (0)	73 (55)	0 (0)	0 (0)	73 (55)	0 (0)	0 (0)	73 (56.6)	0 (0)	0 (0)
Total NCAC species (n=60)	50 (42)	2 (100)	8 (67)	59 (45)	0 (0)	1 (100)	58 (44)	1 (100)	1 (100)	59 (45)	1 (100)	0 (0)	60 (45)	0 (0)	0 (0)	56 (43.4)	0 (0)	4 (100)
***C. tropicalis* (n=45)**	38 (32)	1 (50)	6 (50)	44 (33)	0 (0)	1 (100)	43 (32.8)	1 (100)	1 (100)	44 (33)	1 (100)	0 (0)	45 (33.8)	0 (0)	0 (0)	43 (33)	0 (0)	2 (50)
***C. parapsilosis* (n=7)**	7 (5.9)	0 (0)	0 (0)	7 (5.3)	0 (0)	0 (0)	7 (5.3)	0 (0)	0 (0)	7 (5.3)	0 (0)	0 (0)	7 (5.2)	0 (0)	0 (0)	7 (5.4)	0 (0)	0 (0)
***C. krusei* (n=2)**	0 (0)	0 (0)	2 (17)	2 (2)	0 (0)	0 (0)	2 (1.5)	0 (0)	0 (0)	2 (2)	0 (0)	0 (0)	2 (1.5)	0 (0)	0 (0)	0 (0)	0 (0)	2 (50)
***C. utilis* (n=3)**	3 (2.5)	0 (0)	0 (0)	3 (2.3)	0 (0)	0 (0)	3 (2.3)	0 (0)	0 (0)	3 (2.3)	0 (0)	0 (0)	3 (2.3)	0 (0)	0 (0)	3 (2.3)	0 (0)	0 (0)
***C. glabrata* (n=1)**	1 (0.8)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)
***C. lustaniae* (n=1)**	1 (0.8)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)
***C. pelliculosa* (n=1)**	0 (0)	1 (50)	0 (0)	1 (0.8)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.8)	0 (0)	0 (0)
Total (n=133)	119 (89.5)	2 (1.5)	12 (9)	132 (99)	0 (0)	1 (0.7)	131 (98.5)	1 (0.7)	1 (0.7)	132 (99)	1 (0.7)	0 (0)	133 (100)	0 (0)	0 (0)	129 (97)	0 (0)	4 (3)
***P*-value**		0.023		0.8			0.7			0.8			--------			0.001		

S= Sensitive, SDD: susceptible dose dependent, I: Intermediate, R= Resistant. NCAC: Non-*C. albicans Candida* species

Table 4. Results of disc diffusion testing of nine plant oils against fluconazole-resistant *Candida* isolates (n=12).

Candida species	Oils' mean inhibition zones (mm)± SD								
	Castor	Anise	Lemongrass	Thyme	Peppermint	Clove	Cumin	Cinnamon	Rosemary
C. albicans (n=4)	0 ± 0.00	19.75 ± 6.4	44.5 ± 33.68	51.25 ± 11.8	37.5 ± 17	30.5 ± 1	38.25 ± 23.5	52.5 ± 2.88	22 ± 6.78
C. tropicalis (n=6)	0 ± 0.00	26 ± 13.13	40 ± 10.95	47.16 ± 9.17	54.16 ± 20.35	25.5 ± 5.61	72.16 ± 31.37	45.3 ± 3.26	20.83 ± 5.84

Table 5. Mean of MICs (% v/v) of the four selected natural oils against fluconazole-resistant *Candida* isolates.

***Candida* species**	**Mean of minimum inhibition concentration (MIC) (% v/v)**			
	Cinnamon	**Lemon-grass**	**Thyme**	**Cumin**
***C. albicans* (n=4)**	0.06	0.12	0.12	>2
***C. tropicalis* (n=6)**	0.06	0.12	0.12	>2
***C. krusei* (n=2)**	0.09	0.06	0.12	>2

Figure 1. The distribution of *Candida* species among different age groups.

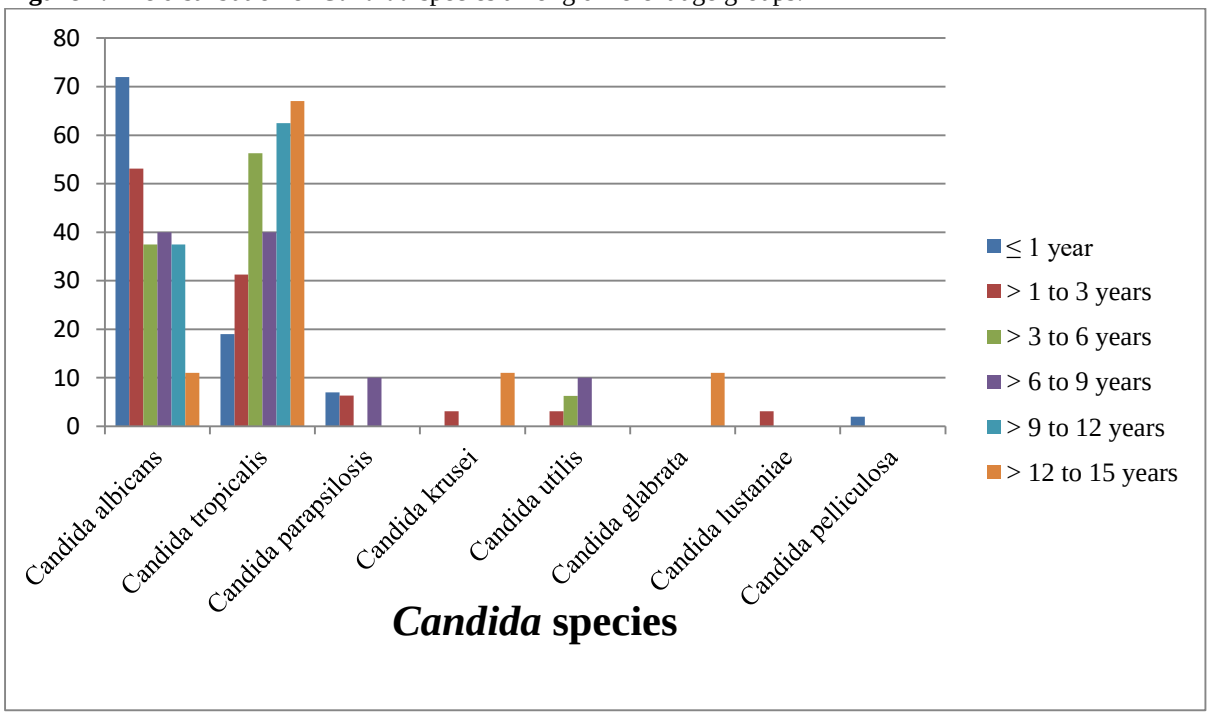
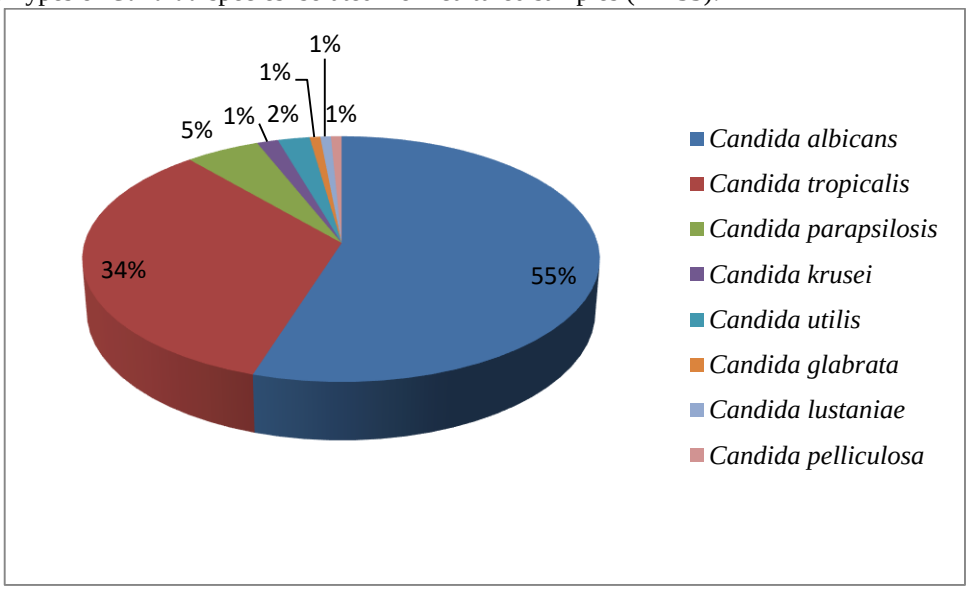


Figure 2. Types of *Candida* species isolated from cultured samples (n=133).



Discussion

Invasive candidiasis is regarded as one of the life-threatening infections, among vulnerable population, particularly children [2]. In this study, *Candida* species were isolated at a rate of 6.27% which was comparable to rates reported in other studies conducted on the pediatric population [17, 18]. A previous study in Egypt demonstrated that the increased incidence of pediatric *Candida* infections was related to the immaturity of their immune system and other risk factors like low birth weight, a pre-term labor and invasive intervention [19].

The wards and ICUs had significantly higher rates of *Candida* isolation than outpatients (*p:* 0.004) and the majority of *Candida* isolates were obtained from patients aged ≤ 1 year old. This finding is consistent with other studies in which the majority of *Candida* were isolated from neonatal ICU patients younger than one-year-old [2, 20].

Our study demonstrated a significant distribution of *Candida* species among various clinical samples ($p = 0.018$). Urine samples were the most common source for *Candida* isolation (67.7%), which agrees with several studies that revealed a predominance of candiduria among different *Candida* infections at rates ranging from 43.3% to 56.4% [21,22]. In the same context, another study recorded that urine was the main source for *Candida* species isolation [23]. However, in other studies, bloodstream candidemia was the most prevalent fungal infection, which can be attributed to the fact that these studies were conducted on hospitalized patients with poor health conditions [24, 25]. This was no far from our study, as blood samples were found as the second common source for isolation of *Candida* species and might be related to the high vulnerability of our study population to the risk of candidemia.

In our study, *C. albicans* was recovered at a higher rate (54.9%) than NCAC species (45.1%), which agrees with other studies that reported the predominance of *C. albicans* over NCAC species [4,22]. However, a previous study in Egypt reported that NCAC species have predominance over *C. albicans* species [19], which aligns well with recent reports on the universal shift in the trend of *Candida* toward the non-albicans species with rates ranging from 51.42% to74.4% [2, 5, 26]. The higher predominance of *C. albicans* over NCAC species in our study may be attributed to that the majority of the isolates were recovered from urine and blood samples, where *C. albicans* has strong ability of biofilm formation and adherence to indwelling devices (central venous and urinary catheters) [21]. *C. tropicalis* and *C. parapsilosis* were the most prevalent non-albicans *Candida* species in the present study, which was consistent with several previous studies [15, 27-29].

In this study, the resistance rate to fluconazole (9%) among *Candida* isolates was observed to be higher than other antifungal drugs. This coincides with universal reports and can be owed to the selective fluconazole pressure, since it is the most widely used antifungal in treating *Candida* infections [4]. Our result is concordant with several studies that reported fluconazole resistance at rates of 10.7%, 13.3%, 11.05% and,

10.5%, respectively [1, 2, 21, 26]. Other studies reported higher fluconazole resistance rates of 52.7%, 54.5%, 31% and 34.8%, which might be related to variable study populations and underlying risk factors for emergence of resistance [3, 4, 30, 31]. In our study, fluconazole resistance was observed at a significantly higher rate among NCAC than albicans species, including the intrinsically resistant *C. krusei* (*p*: 0.023). *Candida tropicalis* was the most resistant species to fluconazole followed by *C. albicans* which complies well with several previous reports [3, 4, 21, 30, 31]. In concordance with previous studies, the majority of *Candida* isolates in the present study were susceptible to the remaining antifungals tested, with no resistance recorded for micafungin or amphotericin B [1, 2].

By testing the in-vitro efficacy of natural oils against fluconazole- resistant *Candida* in our study, cinnamon, thyme, and lemongrass were the oils with the highest efficacies against *Candida* isolates. These findings agree with several other studies' reports on oil's antifungal activity [6, 32-35]. Our study revealed that castor oil had no antifungal effect against all tested isolates by disk diffusion method. Consistently, another study classified castor as an oil of weak potency by disc diffusion and broth micro-dilution methods [8]. Several factors may contribute to variations in the MICs of essential oils among different studies, such as the method of agar or broth micro dilution, the solubility of oil, and microbial exposure to the plant oil [36]. Several studies highlighted the potential role of natural plant oils as effective therapeutic compounds with low toxicity, anti-inflammatory and significant antifungal efficacy, particularly among fluconazole-resistant *Candida* [6, 33]. These natural oils can cause cell death by attacking the fungal cell membrane, causing leakage of their contents, also, can inhibit cellular respiration and fungal proliferation. These deleterious effects of oils are attributed to their alcoholic, phenolic and terpenoid components that have potent antimicrobial activity [6].

In the era of increasing global reports on *Candida* infections and the emergence of antifungal resistance, expanding the knowledge of the latest trends of common *Candida* species and their antifungal resistance profiles is vital to guide appropriate antimicrobial stewardship programs and enable clinicians to make informed therapeutic decisions [37, 38]. Our findings regarding the *in-vitro* efficacy of natural oils against drug-resistant *Candida*, in conjunct to data reported by previous studies, may open horizons for development of new therapeutic options for treatment of *Candida* infections and overcome the rising problem of resistance to current antifungal agents. However, there is still a room for future researches to further explore the mechanisms of action of plant oils against *Candida* spp., and perform in-vivo trials using natural oils to reveal their therapeutic clinical activity.

Conclusion

Candida albicans was more prevalent than NCAC species in all types of samples. The most frequently isolated NCAC species were *C. tropicalis*, and *C. parapsilosis.* Fluconazole recorded a total resistance rate of 9% with a significantly higher incidence among NCAC species than *C. albicans*. Cinnamon oil showed the best in-vitro efficacy against fluconazole-resistant isolates.

Author contribution

HHA, YMS and AMM designed the research. HHA conducted the practical work and wrote the original paper draft. NSS supervised practical work, data analysis. NSS and MAI conducted manuscript review. All authors read and approved the manuscript.

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None.

Data availability

Data generated or analysed in this study are available in this article.

Conflict of interest

All authors declare no conflict of interest.

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