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Characterization, Virulence Factors and Antifungal Susceptibility of Vulvovaginal *Candida* Isolated from Women at Qena, Egypt

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ALTHOUGH the incidence of vaginitis caused by non-*albicans Candida* tends to be increased, *C. albicans* still the main causative agent of vaginitis *Candida*. Eighty-eight vaginal swab samples were collected from women with acute vaginitis in Qena, Egypt. Of 50 isolates, 39 admitted into *C. albicans* and 11 non-*albicans Candida* isolates (78% and 22% prevalence, respectively) were identified. Youths were more susceptible to infection with vulvovaginal *Candida*, the rate of infection decreased with increase education levels and the risk of infection was greater among douching use women. All isolates belonging to *Candida* taxa were positive to proteinase activity and 48 (96%) were lipase producers. Non-*albicans Candida* (*C. glabrata*, *C. tropicalis* and *C. krusei*) were more proteinase producers than *C. albicans* (P< 0.000). Compared with *C. tropicalis*, the other isolated *Candida* exhibited less lipase activity (P< 0.000). The higher lipase capacity of *C. tropicalis* may reflect their increased prevalence among non-*albicans Candida* group. Among five essential oils, cinnamon and clove oils showed strong efficacy against isolated *Candida* strains compared with miconazole antifungal.

Keywords: Vaginitis, Conventional, *Candida*, Virulence factors, Antifungal susceptibility.

Introduction

Vaginal infection is the common disease affects the wide sector of women worldwide, especially during childbearing age. The second causative agents of this infection after bacteria are *Candida* (Achkar & Fries, 2010). *Candida* species are part of normal microbiota of several regions of the human body including vagina (Shao et al., 2007). The type and prevalence of *Candida* spp. recovered from women with vaginitis depending mainly on the population site and ecological conditions (Galan-Ladero et al., 2009). In African countries, *C. albicans* is the common species (78.3-96.1%) isolated from vaginitis, followed by *C. glabrata* (3.9-12%) and *C. tropicalis* (3.5-5.4) (Konate et al., 2014 and Shaaban et al., 2015). The incidence of vaginitis caused by non-*albicans Candida* spp. tend to increase (Majumdar et al., 2016). Recently in Burkina Faso, Sangaré et al. (2018) reported the prevalence of non-*albicans Candida* species, including *C. glabrata* (32.69%) and *C. tropicalis* (15.38) and *C. krusei* (11.54) from the isolated *Candida*.

Different expression levels of virulence factors were observed among different *Candida* species (Mane et al., 2012). The virulence factors that contribute to pathogenesis include the production of various exoenzymes. Extracellular proteases and lipases play the fundamental role in the adhesion and invasion of cell membrane enabling penetration of the tissue (Staniszewska et al., 2012 and Mayer et al., 2013). The expression level of different enzymes in vaginitis may affect the disease severity (Haynes, 2001). Recently, in Iran, Fatahinia et al. (2017) conducted that activities of proteinase and phospholipase in non-*albicans Candida* species (*C. glabrata* and *C. krusei*) were found to be lower than the *C. albicans* species complex including *C. albicans* and *C. dubliniensis*.

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Several literatures were documented the side effects of synthetic antimicrobials. Moreover, during 2006-2013 it has been observed that the susceptibility of different *Candida* species to azole treatment has decreased (Wang et al., 2016). Therefore, attention has been increasing to alter synthetic preservatives with natural, effective and nontoxic compounds (Smid & Gorris, 1999). The essential oils contain a variety of volatile molecules including terpenes, terpenoids, aldehydes, ketones, lactones and phenolic compounds which have antifungal consequences (Bakkali et al., 2008 and Akthar et al., 2014). Several studies have been emitted to clarify the effect of essential oils and their main compounds on different *Candida* species (Benlafya et al., 2014; Radwan et al., 2014 and Karo et al., 2017).

Therefore, this work aimed to identification of vulvovaginal *Candida* isolated from women at Qena, Egypt, evaluate some virulence factors and assessment the susceptibility of *Candida* spp. to essential oils.

Materials and Methods

Samples collection

Eighty-eight vaginal samples collected from women aged from 16-60 years complaining from vaginal infection at Center of Obstetrics and Gynecology (Dr. Safaa Adly) Qena, Egypt. Samples were taken from patients using sterile spectrum and sterile cotton swabs with long handle. The swabs were immediately covered by its sterile cover and transported to the laboratory for quick examination and culturing (Shaaban et al., 2015).

Direct microscopic examination and samples culturing

Distinctive features of yeasts can be determined by observing the morphology. Microscopes can be used to rapidly identify and detect possible yeasts in the clinical sample slide that was prepared from each vaginal swab and was examined under the light microscope on power 40x and 100x. Culturing of samples was obtained by shaking well the sterile swab of each vaginal sample in sterilized distilled water and poured in a sterile plate followed by addition 20ml of Sabouraud Dextrose Agar (SDA). Plates were incubated at 37ºC for 96hr to appear colonies.

Conventional identification

Germ tube test

Germ tube is a quick check of *Candida albicans* and *Candida dubliniensis*. Germ tube appeared as extending outgrowth from the yeast cells (Ellis et al., 2007) by inoculation one or two colonies of culture suspected *Candida* with 0.5ml of human serum, which contains 0.5% of glucose in the Eppendorf tube and incubated at 37ºC for 2-3hr. After required incubation time, a complete loop of the culture on a glass slide and overlaid with a sliding lid and examined microscopically for the presence or absence of the formation of the germ tube.

Growth on HiChrome Candida differential agar

HiChrome agar is a differential and selective medium, for rapid differentiation of *Candida* species namely *C. albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis* according to coloration and colony morphology (Mahajan et al., 2014).

Molecular characterization of selected strains

Based on HiChrome differential agar medium results, 4 strains selected randomly (one from each *Candida* species) to further identification. Yeast strains were cultured on Sabouraud Dextrose Broth for 2 days at 30°C. DNA was extracted as previously described (Robert et al., 1995). Internal transcribed spacer ITS 1– 5.8S rDNA–ITS 2 fragment of yeast species were amplified using universal primer pair ITS1 and ITS4 (ITS1: 3'-TCCGTAGGTGAACCTGCGG-5', ITS4: 3'-TCCTCCGCTTATTGATATGC-5') (White et al., 1990), with the following amplification conditions: 95ºC for 15sec. followed by 30 cycles of 95ºC for 20sec, 50ºC for 40sec and 72ºC for 1min, with a final extension step at 72ºC for 5min. PCR fragments were detected by 1.2 agarose gel electrophoresis and visualized on a UV transilluminator. Purification of PCR product was done using SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) and sequenced. Sequences were edited with Chromas Lite (Technelysium Pty. Ltd.). The resulted sequences were blasted against GenBank to identify the selected isolates to species level. Phylogenetic analysis was done with the help of software mega 4.0 (Tamura et al., 2007) using *Saccharomyces cerevisiae* DQ674252 as outgroup.

Some virulence factors (Enzyme activity)

This study focused on identifying the activity of extracellular enzymes of different isolates of

vulvovaginal *Candida*. Different isolated taxa were subjected to qualitative analysis of proteinase and lipase enzymes. The activities of proteinase were tested on Casein hydrolysis medium (Paterson & Bridge, 1994). The medium was distributed into 15 ml test tubes (10ml/tube), autoclaved at 121ºC for 20min. Tubes were inoculated with 50µl from spore suspensions ($1x10^5$/ml) and incubated at 37ºC for 7 days. Positive results were taken by degradation of casein protein as clear depth in the tube. Proteinase activity expressed as; weak (1-9mm), strong (10-19mm) and very strong producers (≥20mm).

The production of lipase was tested on Ullman & Blasins (1974) medium. As described before, tubes with medium autoclaved, inoculated by different isolates and incubated for 7 days at 37ºC. The lipolytic activity was detected as a visible white precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme. The depth of each visible precipitate (in mm) was measured. Lipase activity expressed as; weak (1-4mm), strong (5-14mm) and very strong producers (≥15mm). The enzymes activities were performed in triplicate.

Antifungal susceptibility

Five essential oils (Black seed, Cinnamon oil, Clove oil, Coconut oil, and Lemon oil) were tested against different *Candida* isolates. One ml of the above spore suspension was transferred into petri dish followed by addition of about 20ml liquefied SDA medium. After solidifying, 6mm wells were made in each plate and inoculated with 25µl of each oil. Plates were incubated at 37ºC for 48hr. Miconazole (0.01g/ml) was used as positive control and the average inhibition zone was measured.

Statistical analysis

all obtained data were statistically analyzed by one-way ANOVA and Post Hoc Test (LSD). $P< 0.05$ was considered statistically significant.

Results

Conventional and molecular identification of Candida spp.

Eighty-eight cases of women complaining of fungal vaginitis were studied. All vaginal swabs from women were exposured directly to direct microscope examination (DME), which revealed that, 35 samples (39.8%) of patients were positive showing budding cells and pseudo hyphae. 56.8% (50 out of 88) of vaginal swabs were positive on SDA medium producing white to cream colonies. The positivity of culturing on SDA medium is greater than DME (Fig. 1).

Germ tube is the most important criteria to differentiate *albicans* group (*Candida albicans* and *Candida dubliniensis*) from other *Candida* species. The formation of germ tube took 2hr after inoculation in human serum at 37°C. Thirty-nine (78%) of isolates were positive for germ tube test (*Candida albicans* or *Candida dubliniensis*) and eleven (22%) isolates were negative for germ tube (non-*albicans* group). In relation to the colony colors of the various yeast taxa grown on HiChrome agar, 39 (78%) of taxa identified as *C. albicans* (appeared as light green colored smooth colonies), 8 (16%) as *C. tropicalis* (blue colonies), two (4%) taxa as *C. glabrata* (Creamy white), and the final one as *C. krusei* (pink fuzzy) (Fig. 2).

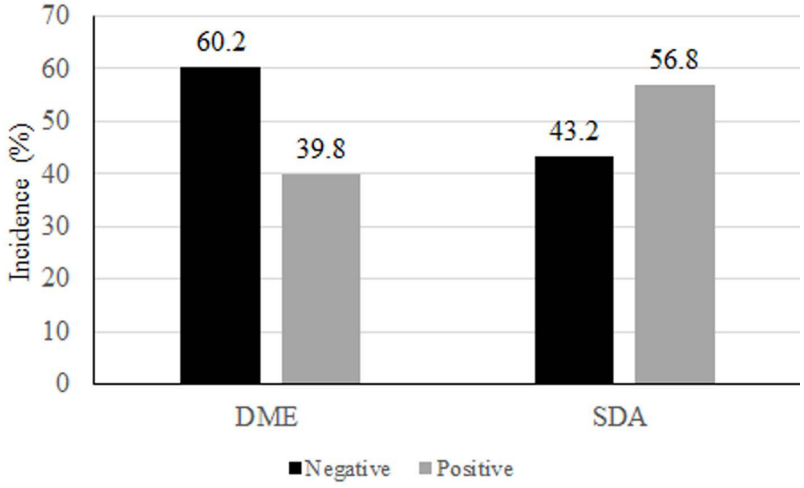

Fig. 1. Positive and negative (%) samples by direct microscopic examination (DME) and grown on SDA medium.

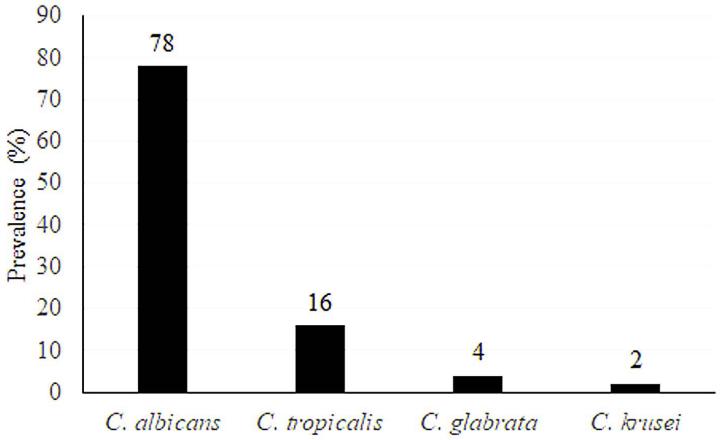

Fig. 2. Prevalence (%) of different isolated *Candida* spp.

The resulted sequences from four selected isolates were edited and deposited in GeneBank with accession numbers MG707638, MG707639, MG707640 and MG707641. Comparison of ITS sequences with sequences available in the GenBank nucleotide database indicated that the DNA sequences from selected yeast isolates had 99-100% sequence identity with yeast species sequences from GenBank. Phylogenetic tree was generated from tested four sequences with closely related yeasts species from GenBank indicated that the selected strains formed distinct four clades; MG707638 strain clustered with *Candida albicans* strains recovered from different places and MG707639 grouped with *Candida tropicalis* with while MG707640 and MG707641 forming clades with *Candida glabrata* and *Pichia kudriavzevii* (teleomorph *C. krusei*) with 92-100% bootstrap values (Fig. 3).

Prevalence of Candida taxa in women patients

The age range of women patients was 16-60 years, with mean 28.1±9.43 years. Most of *C. albicans* (69.2%), *C. tropicalis* (75%) and *C. krusei* (100%) infections were diagnosed in women aged from 21-34 years. In general, the rate of vaginal infection with *Candida* decrease with increasing the academic qualifications; most of the infected women were illiterate and low education levels by 36% and 38%, respectively, but were for secondary (12%) and university (14%). Of the 50 patients, 38 (76%) were admitted into douching use women and 12 (24%) into douching non-using women. Approximately 37 (74%) of the women patients were not using any method of contraception. The frequency of vaginal infection performed 10% by the first time, 28% by one of the year and 62% by more than one in the year (Table 1).

Virulence factors

The abilities of different isolates to produce virulence factors including proteinase and lipase were studied. The obtained results clarified that, all of *Candida* isolates are positive to proteinase activity and 48(96%) were lipase producers. The mean enzyme activities for proteinase and lipase ranged from 7.5±0.35 - 62±0.58 and 10±0.58 - 16.5±0.19, respectively.

About 23% (9 out of 39 isolates) of *C. albicans* showed strong activity for protease production, while the remaining (77%) exhibited weak activity. Exactly half of *C. tropicalis* isolates showed very strong proteinase production and the other one was strong in its production. The remaining isolates of *C. glabrata* and *C. krusei* were very strong producers (Fig. 4). In relation to lipase, 41% of the *C. albicans* isolates were very strong in lipase production, 53.8% were strong, and the remaining two isolates were non-producers. All of *C. tropicalis* isolates were very strong producers. The isolates belonging to *C. glabrata* and *C. krusei* exhibited strong activity (Fig. 5).

The resulted analysis for proteinase and lipase activities revealed a significant difference between different species of *Candida* ($P< 0.000$). Compared with *C. albicans*, non *albicans Candida* (*C. glabrata*, *tropicalis* and *krusei*)

produced significant more protease (P< 0.000). Non-significant was observed in proteinase production between *C. glabrata* and *C. krusei* (P= 0.689). On the other side, *C. tropicalis* and *C. krusei* produced significant more and fewer lipase activities (P< 0.000) respectively than *C. albicans*. There was no significant difference in lipase activity produced by *C. albicans* and *C. glabrata* (P= 0.244) (Table 2).

Antifungal susceptibility

Among five tested essential oils, all tested *Candida* species showed strong antifungal susceptibility to cinnamon and clove oils, but in case lemon oil, *C. albicans* and *C. krusei* were affected. The other remaining oils (black seed and coconut oil) did not show any detectable effect against the tested isolates. Compared with miconazole as control, cinnamon and clove oils showed significantly higher efficacy against tested isolates, except in case of cinnamon with *C. glabrata* was less efficiency. The susceptibility of *C. albicans* to essential oils was greater than non-*albicans Candida* (Table 3).

Discussion

Vaginal candidiasis is a common vaginal infection attributed to different species of *Candida* of 88 symptomatic women, 50 (56.8%) were diagnosed as vaginal candidiasis infection with a higher prevalence rate than that reported by several researchers (Paulitsch et al., 2006; Yusuf et al., 2007 and Ogouyèmi-Hounto et al., 2014). In this work, *C. albicans* was the predominant cause of vaginal candidiasis, followed by *C. tropicalis*, *C. glabrata* and *C. krusei*. Although many literatures have illustrated a shift towards an increase in non-*albicans Candida* species (Dan et al., 2002; Fatahinia et al., 2017 and Sangaré et al., 2018). This result collaborating with those obtained by Jasim et al. (2016), they reported the prevalence of *C. albicans*, *C. tropicalis* and *C. glabrata* were 78%, 14% and 2% from different clinical specimens, respectively. In Egypt, Shaaban et al. (2015) indicated that the most prevalent vaginal *Candida* species was *C. albicans* (78.3%) followed by *C. glabrata* (12%) then *C. tropicalis* (5.4%). In most regions of the world, *C. glabrata* was the common taxa among non-*albicans* vulvovaginal *Candida* (Paulitsch et al., 2006; Konate et al., 2014 and Ameen et al., 2017).

Recently, several researches were done to clarify the prevalence of different vulvovaginal *Candida* to age, educational level, work, personal hygiene and contraceptive method of the women (Yusuf et al., 2007; Rezaei-Matehkolaei et al., 2016 and Swaminathan et al. 2017). The current study showed that the maximum incidence of vaginal candidiasis was found between youths (20-34 age), the rate of vaginitis was decreased by increasing educational level and the douching using women were more susceptible to the risk of infection with vaginitis *Candida*. This finding is in full agreement with data obtained by Hassan et al. (2017) they concluded that 71% of vaginal infection in Egypt were admitted into women aged 19-30 years. The reason for the high incidence rate in this age group includes increased sexual activity and a new effect of reproductive hormones (Sobel et al., 1998). The vaginal douching increases the infection rate due to change in normal flora, but not affect the type of *Candida* species (La Ruche et al., 1999 and Shaaban et al., 2015).

In our work, protease activity was seen in 100% of different taxa with the more active producer belonging to non-*albicans* group (*C. glabrata*, *C. tropicalis* and *C. krusei*). The correlation between the taxa and proteinase was significant, reflecting that non-*albicans* produced the highest levels of proteinase activity. These results are partially agreed with Moharram et al. (2013) they showed that protease was produced by 83 (89.2%) out of 93 isolates tested with active isolates belonging to *C. albicans* and *C. krusei*. The opposite finding was reported by de Melo Riceto et al. (2015) who detected the proteinase activity in *C. albicans*, *C. tropicalis* and *C. parapsilosis* isolates but not in *C. glabrata* and *C. krusei*.

Candida albicans revealed more phospholipase activity than non-*albicans Candida* species (Chin et al., 2013; de Melo Riceto et al., 2015 and Fatahinia et al., 2017), while in the present work *C. tropicalis* strains showed the highest level of lipase among *albicans* and non-*albicans Candida* which corroborate with results obtained by Thangam et al. (1989) and Moharram et al. (2013) they reported high lipase activity in *C. tropicalis*. Generally, the microorganism that produces lipolytic enzymes might have abilities to lysing competing microflora and have a protective role against host by suppressing the cellular and humoral responses which reflecting the increase in their incidence (Stehr et al., 2003 and Toth et al., 2017).

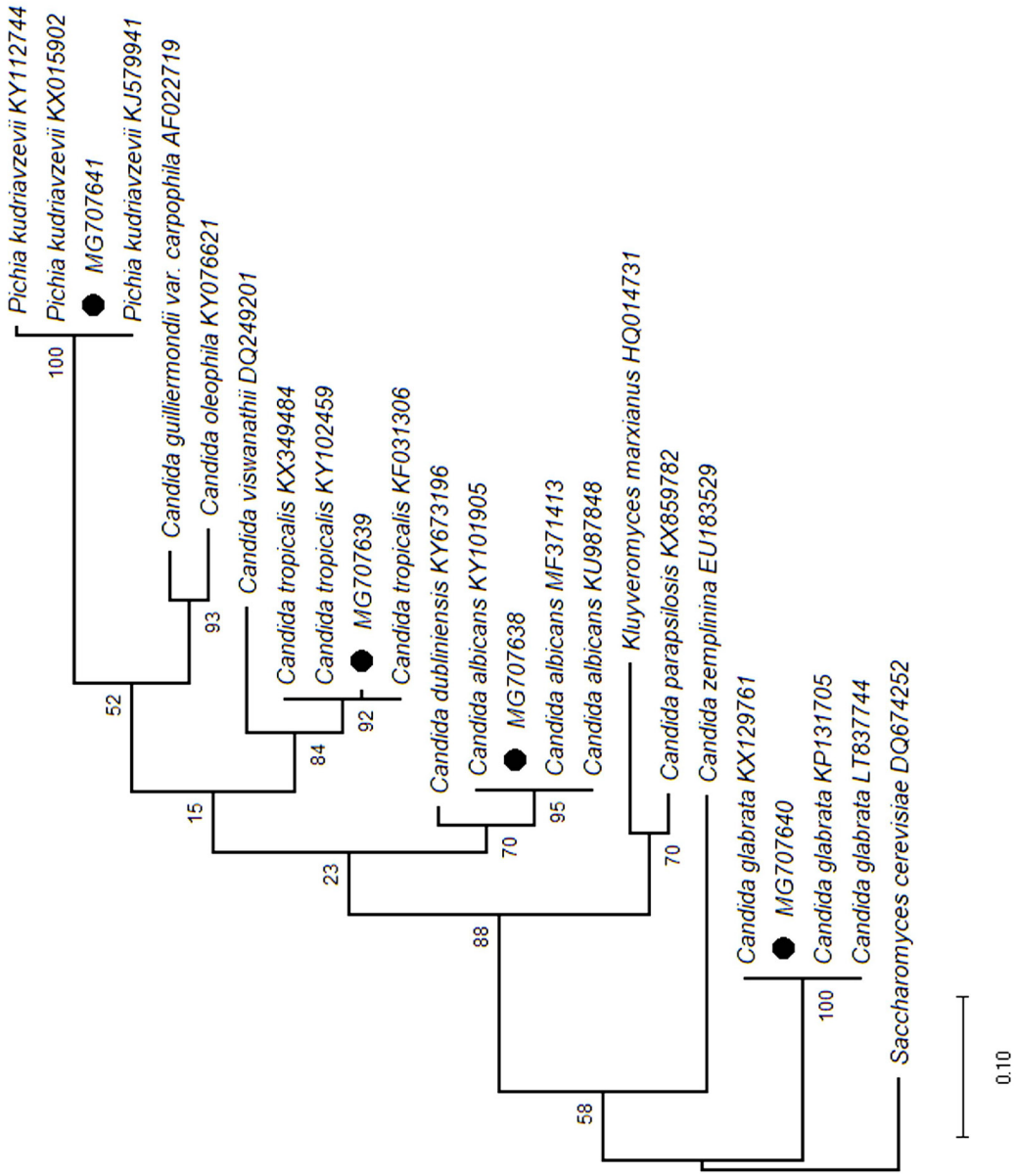

Fig. 3. Maximum likelihood tree based on the rDNA-ITS sequences data for 4 isolates of yeast. The tree was rooted with *Saccharomyces cerevisiae* DQ674252 as the outgroup.

TABLE 1. Demographic data of the women affected by vulvovaginal *Candida*.

Patients criteria	*C. albicans* n= 39	*C. tropicalis* n= 8	*C. glabrata* n=2	*C. krusei* n= 1	Total n= 50
Age					
≤20	5 (12.8)	1(12.5)	1(50)	-	7 (14)
21-34	27 (69.2)	6 (75)	-	1(100)	34 (68)
≥35	7 (18)	1(12.5)	1(50)	-	9 (18)
Academic Qualifications					
Illiterate	12 (30.8)	5 (62.5)	1(50)	-	18 (36)
Primary	16 (41)	2 (25)	-	1(100)	19 (38)
Secondary	5 (12.8)	-	1(50)	-	6 (12)
University	6 (15.4)	1(12.5)	-	-	7 (14)
Personal hygiene					
Douching use	30 (76.9)	6 (75)	1(50)	1(100)	38 (76)
Non-use	9 (23.1)	2 (25)	1(50)	-	12 (24)
Contraceptive method					
Non-use	29 (74.4)	6 (75)	1(50)	1(100)	37 (74)
IUD	3 (7.7)	-	-	-	3 (6)
Oral contraceptives	4 (10.2)	1(12.5)	1(50)	-	6 (12)
Injectables	3 (7.7)	1(12.5)	-	-	4 (8)
Frequency of vaginal infection					
First time	4 (10.2)	1(12.5)	-	-	5 (10)
One in year	9 (23.1)	3	1(50)	1(100)	14 (28)
More than one in year	26 (66.7)	4 (50)	1(50)	-	31 (62)

IUD: Intra Uterine Device

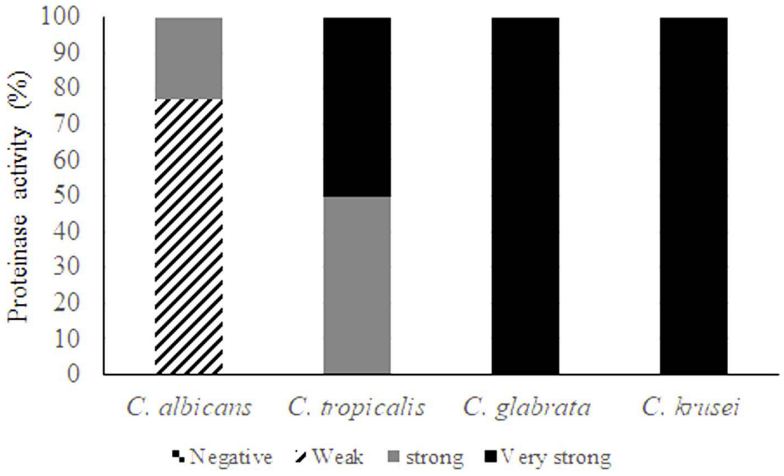

Fig. 4. Proteinase production by *Candida* species.

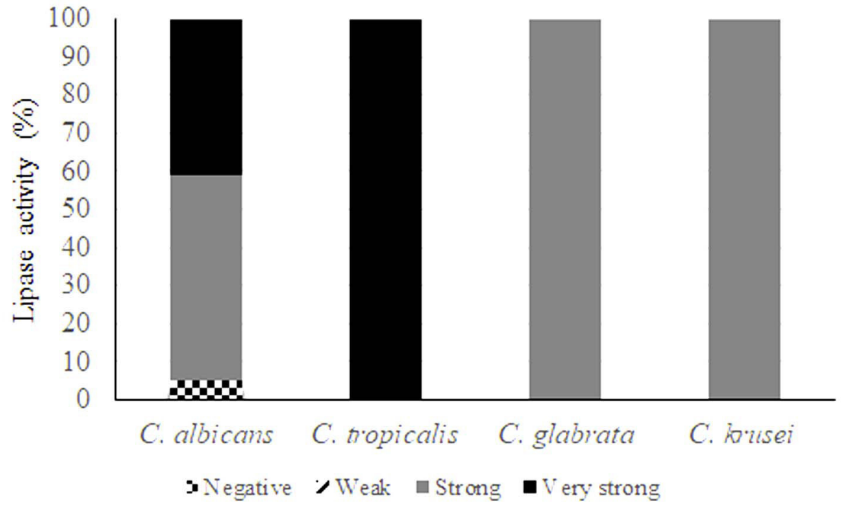

Fig. 5. Lipase production by *Candida* species.

TABLE 2. Enzymatic activities indicated by different *Candida* taxa (in mm).

Virulence attribute	*Candia* species	E I (%)	Range	EA Mean
Proteinase	*C. albicans* (n= 39)	100	4-12	7.5±0.35
	C. tropicalis (n= 8)	100	17-21	19.1±0.48*
	C. glabrata (n= 2)	100	59.5-61	60.2±0.44*
	C. krusei (n= 1)	100	61-63	62±0.58*
Lipase	*C. albicans*	94.8	0-16	13±0.53**
	C. tropicalis	100	16-17	16.5±0.19
	C. glabrata	100	12-13	12.5±0.29**
	C. krusei	100	9-11	10±0.58**

EI= Enzyme index, EA= Enzyme activity.
*: Significant difference between *C. albicans* and other non-*albicans Candida* taxa.
**: Significant difference between *C. tropicalis* and other *Candida* taxa.

TABLE 3. Antifungal susceptibility of *Candida* isolates against different essential oils.

Antifungal	*C. albicans*	*C. tropicalis*	*C. glabrata*	*C. krusei*
Miconazole	21±0.15	19±0.35	24±0.10	18±0.21
Black seed oil	ND	ND	ND	ND
Cinnamon oil	34±0.50*	24±0.43*	22±0.25*	25±0.38*
Clove oil	42±0.22*	27±0.30*	28±0.25*	28±0.22*
Coconut oil	ND	N.d	ND	ND
Lemon oil	18±0.27*	N.d	ND	15±0.34*

*: Significant difference between essential oils and miconazole (positive control).
N.d= Not detected.

The resistance *Candida albicans* and non-*Candida albicans* species isolated from patients, against antifungal agents has increased. Several studies have shown that clove and cinnamon oils had a strong and inhibitory activity against different *Candida* species (Aneja & Joshi, 2010; Fani & Kohanteb, 2011 and Radwan et al., 2014). In the same situation, the combination

cinnamon with clove oils leading to enhancement of the antifungal activity in all cases (Horváth et al., 2016). Omran & Esmailzadeh (2009) indicated that lemon essential oil has a low inhibited effect on different *Candida* species. The chemical composition, structure and functional groups of the oils play a fundamental role in determining their antimicrobial activity (Omidbeygi et al., 2007 and Yesil Celiktas et al., 2007).

Conclusions

Candida albicans is the predominant cause of vaginitis *Candida* in Upper Egypt. Compared with *Candida albicans*, non *albicans Candida* was more producer to virulence factor. The increase in the prevalence of *C. tropicalis* among non-*albicans* species may result from the highest activities of hydrolytic enzymes especially lipase which secreted by this taxon. Cinnamon and clove oils could be considered as the excellent source for new antifungal production.

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توصيف وعوامل الإصابه وقابليه الكانديدا المهبليه المعزوله من النساء فى قنا بمصر للمضادات الفطريه

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يستهدف هذا البحث عزل وتعريف الكانديدا المهبليه وقدرتها على انتاج انزيمى البروتيز والليبيز وقابليه هذه الأنواع لخمسه من الزيوت النباتيه.

تم جمع 88 عينه من النساء اللاتى تم تشخيصهم اكلينيكا يعانون بالإصابه بالفطريات المهبليه فى مدينه قنا. قد اظهرت نتيجه الفحص المباشر للعينات وكذلك المزارع الفطريه لها أن 56.8% من الحالات توكد الأصابه بالكانديدا. تم عزل 50 عزلة من الخمائر منهم 39 (بنسبه 78%) عزله كانديدا البيكانس و11عزله من الكانديدا غير البيكانس وهم 8 كانديدا تروبيكاليس، 2 كانديدا جلابراتا وعزله واحده من كانديدا كروزى. وقد اوضحت النتائج ان 68% من الحالات المصابه بانواع الكانديدا كانت بين الشباب الاتى تتراوح اعمارهن من21 إلى 34 عام، كذلك معدل الأصابه يقل بصفه واضحه بزياده المستوى التعليمى وأن استخدام الغسول المهبلى وعدم استخدام وسائل منع الحمل يرفع فرص الأصابه بالأنواع المختلفه من الكانديدا.

كما اظهرت النتائج أن جميع العزلات لها القدره على انتاج الأنزيمات المحلله للبروتين وأن 48عزله بنسبه (96%) استطاعت انتاج الأنزيمات المحلله للدهون، كان هناك انخفاض معنوى فى معدل انتاج البروتيز بواسطه كانديدا البيكانس عن انواع الكانديدا الأخرى وكذلك اظهرت كانديدا تروبيكالس انتاجيه عاليه معنويا من الليبيز عن جميع انواع الكانديدا الأخرى. كما اوضحت النتائج ان زيت القرفه و القرنفل يؤثرون على جميع انواع الكانديدا المختبره وان كانديدا البيكانس كانت اكثر تاثرا من كانديدا غير البيكانس.