

Original Research Paper

Effect of Thyme, Clove and Cinnamon Essential Oils on *Candida Albicans* and Moulds Isolated from Different Sources

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Abstract: In this study, mycological examination were conducted on different samples from human, animals and poultry in El-Fayoum and Beni-Suef governorates, Egypt in the period from January to June 2013. The antifungal activities of thyme, clove and cinnamon oils against the recovered fungal isolates were tested using agar dilution method. A total of 209 samples were collected (including vaginal swabs from 18 women, 82 cows and 9 buffaloes beside 100 swabs from broiler chickens; 20 crops and 80 proventriculus). Eighty nine fungal isolates were recovered (40 mold and 49 yeast isolates). Eleven (61.1%) fungal isolates were recovered from women; of which 1 isolate was *A. flavus* and 10 were *Candida* species while 39 (42.9%) fungal isolates were recovered from animals; of which 36 were molds and 3 were *C. albicans*. In broiler chickens, 39 (39%) fungal isolates were recovered; of which 36 were yeast; 34 *Candida* species and 2 *Cryptococcus* species and 3 were molds; 2 *A. niger* and 1 *A. flavus*. PCR assay using oligonucleotide primer that amplifying 172bp fragment in *SAP3* gene of *C. albicans* confirmed morphological, biochemical and biological identification of *C. albicans*. The antifungal activities of the tested essential oils against the recovered fungi revealed that thyme oil completely inhibited the growth of different fungal isolates at concentrations of 0.25, 0.5 and 1%. Clove and cinnamon oils completely inhibited the growth of different fungal isolates at a concentration of 6%.

Keywords: *Aspergillus* Species, *C. Albicans*, Thyme Oil, Clove Oil, Cinnamon Oil

Introduction

Fungal infections have been increased in recent years due to a growing number of high-risk patients, particularly immunocompromised hosts (Pinto *et al.*, 2009). Moreover, the mortality rate due to invasive aspergillosis increased to 35% between 1980 and 1997 in the USA (McNeil *et al.*, 2001). Unfortunately, few anti-fungus medicines are available for treating fungus infections, not to mention that most of them have serious side effects (Wang *et al.*, 2012).

Mycotic abortion is the most important consequence of fungal infection of the genital tract, although fungi have been implicated occasionally in other syndromes such as vulvovaginitis or endometritis. The genera of *Aspergillus* and *Penicillium* can grow in a suitable substrate under

appropriate conditions. They can produce toxins which are accounted the majority of abortion cases and can cause metritis in cows (Garoussi *et al.*, 2007).

In poultry, *Candida* species are widely spread throughout the poultry producing areas of the world. In recent years, the growing economic value of poultry has led to the increase of research of poultry diseases. The fungal diseases of poultry have become problematic as bacterial and viral diseases (Darwish, 1989).

In the past, *C. albicans* was assumed to be the only pathogenic yeast of the genus *Candida*. However, it is now known that of the more than 100 species of *Candida*, seven are of medical significance (Hopfer, 1985). *C. albicans* can be found to naturally colonize the skin, buccal mucosa, intestinal tract and vaginal mucosa. It is the primary causative agent of candidiasis, the most

common form of mycotic infection (Wilson and Plunkett, 1970). It was classified as an opportunistic pathogen which increases predominantly in patients with predisposing condition, including immunodeficiencies such as HIV, AIDS, Prolong use of broad-spectrum antibiotics, corticosteroids (Ryan and Ray, 2004) as well as hormonal imbalances, use of oral contraceptives, pregnancy, metabolic and nutritional disorders and poor oral hygiene (Nader-Djalal and Zadeii, 1996) which may affect the normal acidic environment of the vagina, increasing susceptibility to infection. Diabetes and diets rich in carbohydrates may also contribute to chronic yeast infections (De Leon *et al.*, 2002).

Synthetic fungicides are currently used as primary means for the control of fungal disease. In spite of the introduction of new antifungal drugs, they are limited in number. The increase of fungal resistance to classical drugs, the treatment costs and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies (Rapp, 2004).

The uses of plant-derived products as disease control agents have been studied since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Lee *et al.*, 2007). Therefore, there has been increasing interest to replace synthetic preservatives with natural, effective and nontoxic compounds. Those are; in the first place, extracts and essential oils of spices and herbs (Smid and Gorris, 1999). Nowadays, plenty of spices and herbs are valued for their antimicrobial activities and medicinal effects in addition to their flavor and fragrance qualities (Shan *et al.*, 2007).

Aromatic medicinal plants have been used in folk medicine as antimicrobial agents since ancient times (Pinto *et al.*, 2006). It is known that most of their properties are due to their volatile essential oils, extracts and isolated phytochemicals (Carmo *et al.*, 2008). The essential oils from many plants are known to possess antibacterial and antifungal activity (Pinto *et al.*, 2006) but the spectrum of activity and mechanisms of action remain unknown for most of them. Although only limited consistent information exists about activity toward human fungal pathogens, some essential oils have shown important antifungal activity against yeasts, dermatophyte fungi and *Aspergillus* strains (Bakkali *et al.*, 2008), which could predict therapeutic benefits, mainly for diseases with mucosal, cutaneous and respiratory tract involvement (Salgueiro *et al.*, 2004). Previous studies have reported antifungal activity for clove oil and eugenol against yeasts and filamentous fungi, such as several food-borne fungal species (López *et al.*, 2007), human pathogenic fungi (Chaieb *et al.*, 2007) as well as animal pathogens (Ahmad *et al.*, 2005). Clinical experience showed that the efficacy of antimicrobial agents depends not only on their direct effect on a given microorganism but also on the functional activity of the host immune system (Tullio *et al.*, 2012).

The purpose of this study was to identify the phenotypic characters of different fungal isolates recovered from different sources (human, animals and broiler chickens) and to detect the activity of thyme, clove and cinnamon oils against these isolates and possibility for their application in the veterinary medicine.

Material and Methods

Samples

A total of 209 samples were collected (18 vaginal swabs from women admitting to antenatal clinic of the Obstetrics and Gynecology Department, Faculty of Medicine, Beni-Suef University, Beni-Suef, suffering from vaginitis, itching and vaginal discharges and 91 vaginal swabs from animals; 82 cows and 9 buffaloes, suffering from repeat breeding, abortion or different reproductive disorders as well as 100 swabs from the crop or proventriculus of broiler chickens) from different areas in El-Fayoum and Beni-Suef governorates during the period from January to June 2013.

Fungal Isolation

All samples were taken immediately and transferred directly into pre-enrichment broth (Malt Extract broth, Oxoid) and incubated at 37°C for 24-48 h, then cultured on Sabouraud dextrose agar medium (Oxoid) and incubated at 37°C for 24-48 h.

Identification of Fungal Isolates

The recovered fungi were identified morphologically according to Rippon (1988). Mycelial fungi were identified by examination of mycelial morphology, the reverse colour as well as examination of lactophenol cotton blue stained smears. Yeast like fungi were identified by colonial morphology and examination of Gram's stained smears, then they were confirmed by additional biochemical laboratory tests as sugar fermentation tests (glucose, maltose, galactose and lactose) and urease test according to Rippon (1988).

Chlamyospore Production

It was used to identify *C. albicans* using rice extract agar medium which was prepared according to Taschdjian (1953). Yeasts were inoculated onto rice agar according to the method of Rippon (1988) by making 3 parallel cuts about 5 mm apart into agar. A coverslip was laid on the surface of the agar covering a portion of the inoculation streaks. The inoculated plates were incubated at 30°C for 24-48 h and examined microscopically through the coverslip for the presence of hyphae, blastoconidia, chlamydoconidia or arthroconidia.

Germ Tube Test

It was used to identify *C. albicans* using human serum according to Rippon (1988).

Polymerase Chain Reaction

PCR using Oligonucleotide primers that amplify a 172bp fragment in *SAP3* gene of *C. albicans* (Muñoz *et al.*, 2003) was applied on 3 randomly selected isolates; morphologically and biochemically identified as *C. albicans*; one from cow, one from human and one from chicken.

Primers:

- ~ Primer 1 (Forward primer):
- 5'- CTG-ATT-TAT-GGG-TTC-CTG-AT - 3'
- ~ Primer 2 (Reverse primer):
- 5'- CAT-GTC-CCT-TGT-GAA-GTA-GT - 3'

Agar Dilution Method for Detection of Antifungal Activity of Essential Oils

According to the method of Jeff-Agboola *et al.* (2012) the antifungal activity of thyme, clove and cinnamon against 30 randomly selected fungal isolates were done. The tested isolates included 10 *C. albicans* (2, 4 and 4 from animal, human and chickens, respectively), 5 *A. flavus* (3, 1 and 1, respectively), 7 *A. niger* (5 animal and 2 chicken isolates), as well as 2 *A. fumigatus*, 2 *A. nidulans* and 4 *Penicillium* (all from animal). Briefly, the tested fungi were grown on SDA at 35°C for 48 h, then cells were suspended in physiological saline (0.9% NaCl) and the suspension was adjusted to 1×10⁶ CFU. SDA was prepared and autoclaved at 121°C for 15 minutes and kept at 55°C and then the tested oils were sterilized by filtration (pore size, 0.45 µm) and were

mixed with SDA according to the tested concentration. Thyme oil (Herbal Global co.) was prepared at concentrations of 0.25, 0.5 and 1% while, clove and cinnamon oils (Al captain co.) were prepared at concentrations of 0.5, 1, 2, 3, 4 and 6%.

The oil-agar medium (10 mL) was then poured into sterile petri dishes and was solidified. Equal amounts of the fungal suspensions were inoculated and spread onto the agar plates. The plates were then incubated at 37°C for 24h and then examined daily for 8 days.

Results

Identification of Recovered Molds Isolates

Forty molds isolates were recovered during the present study (one from human, 36 from animal and 3 from broiler chickens), which were recognized as 15 *Aspergillus niger*, 10 *A. flavus*, 2 *A. fumigatus*, 3 *A. nidulans* 6 *Penicillium* and one of each of *Stachybotrys corda*, *Gliocladium corda*, *Xylohypha bantia* and lower fungi.

Identification of Recovered Yeast Isolates

Forty nine yeast isolates were recovered during the present study (10 from human, 3 from animal and 36 from chickens), 47 isolates were recognized as *Candida* species which were identified as 29 *C. albicans*, 6 *C. pseudotropicalis*, 6 *C. krusei* and 5 *C. rugosa*, 1 *C. stellatoidea* and 2 isolates were recognized as *Cryptococcus* species; which were identified as *C. neoformans* and *C. laurentii*.

Table 1. Recovery rate of molds and yeasts isolated from human samples

No. of samples	Mycelial Fungi (<i>A. flavus</i>)		Yeasts								Total			
	No.	%	<i>C. albicans</i>		<i>C. stellatoidea</i>		<i>C. krusei</i>		<i>C. rugosa</i>		No.	%		
18	1	5.6	7	38.9	1	5.6	1	5.6	1	5.6	10	55.5	11	61.1

Table 2. Recovery rate of molds and yeasts isolated from animal samples

Source of samples	No. of samples	Mycelial Fungi																Yeasts							
		<i>A. niger</i>		<i>A. flavus</i>		<i>A. fumi-gatus</i>		<i>A. nid-ulans</i>		<i>Penici-llium</i>		<i>S. corda</i>		<i>G. corda</i>		<i>X. bantia</i>		Lower fungi		Total	<i>(C. albicans)</i>		Total		
Cattle	82	13	15.9	8	9.8	1	1.2	3	3.7	5	6.1	1	1.2	1	1.2	1	1.2	1	1.2	34	41.5	2	2.4	36	43.9
Buffaloes	9	0	0.0	0	0.0	1	11.1	0	0.0	1	11.1	0	0.0	0	0.0	0	0.0	0	0.0	2	22.2	1	11.1	3	33.3
Total	91	13	14.3	8	8.8	2	2.2	3	3.3	6	6.6	1	1.1	1	1.1	1	1.1	1	1.1	36	39.6	3	3.3	39	42.9

Table 3. Recovery rate of molds and yeasts isolated from broiler chicken samples

Source of samples	No. of samples	Mycelial Fungi																Yeasts					
		<i>A. niger</i>		<i>A. flavus</i>		<i>C. albicans</i>		<i>Candida</i>				<i>Cryptococcus</i>				Total							
		No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%						
Crop	20	0	0.0	0	0.0	4	20.0	2	10	0	0.0	1	5.0	0	0.0	0	0.0	0	0.0	0	0.0	7	35
Proventriculus	80	2	2.5	1	1.3	15	18.8	4	5	5	6.3	3	3.8	1	1.3	1	1.3	1	1.3	1	1.3	32	40
Total	100	2	2.0	1	1.0	19	19.0	6	6	5	5.0	4	4.0	1	1.0	1	1.0	1	1.0	1	1.0	39	39



Fig. 1. Chlamydospore production on rice agar medium under microscope yeast cells appear as spherical clusters at regular intervals on pseudohyphae and having the chlamydo-spores on the pseudohyphae like shiny pearls



Fig. 2. Germ tube formation of *C. albicans* under microscope. Germ tubes appear as hypha-like extensions of yeast cells, produced usually without a constriction at the point of origin from the cell

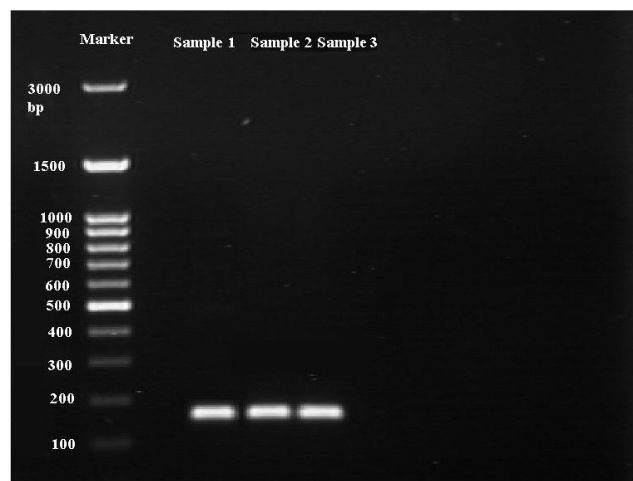


Fig. 3. PCR results for 3 isolates of *C. albicans* from different sources; animal origin (Sample 1), human origin (Sample 2) and chicken origin (Sample 3). Amplification of the 172bp fragment of *SAP3* gene from all tested *C. albicans* isolates which showed a positive amplicons migrates with molecular size of about 172 bp with the molecular DNA size marker



Fig. 4. The antifungal effect of Thyme oil against *A. fumigatus*; recovered from cattle, at a concentration of 0.25% showing complete inhibition of the growth



Fig. 5. The antifungal effect of Clove oil against *C. albicans*; recovered from human, at a concentration of 4% showing constricted growth of the colonies appeared after the 4th day of incubation

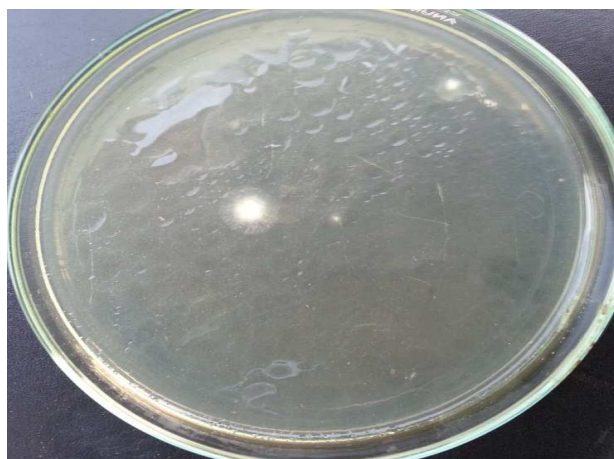


Fig. 6. The antifungal effect of cinnamon oil against *Penicillium* species; recovered from cattle, at a concentration of 2% showing constricted growth of the colonies appeared after the 2nd day of incubation

Table 4. Antifungal effect of Thyme, Clove and Cinnamon oils on different fungal isolates

Essential oil	Conc.	Growth examination				
		1st day	2nd day	3rd day	4th day	5-8th day
Thyme	0.25%	-	-	-	-	-
	0.5%	-	-	-	-	-
	1%	-	-	-	-	-
Clove	0.5%	+	+++	+++	+++	+++
	1%	+	+++	+++	+++	+++
	2%	-	+	++	+++	+++
	3%	-	-	+	++	+++
	4%	-	-	-	+	+
	6%	-	-	-	-	-
Cinnamon	0.5%	+	+++	+++	+++	+++
	1%	+	+++	+++	+++	+++
	2%	-	+	++	+++	+++
	3%	-	-	+	++	+++
	4%	-	-	-	+	++
	6%	-	-	-	-	-

- : No growth, +: Mild growth, ++: Moderate growth, +++: Heavy growth

Prevalence of Fungal Infections in Human, Animals and Broiler Chickens

In human, 11 fungal isolates were recovered with a recovery rate of 61.1% of which 1 (5.6%) was *Aspergillus flavus* and 10 (55.5%) were *Candida* species which were identified morphologically, biochemically and biologically as 7 *C. albicans*, 1 *C. stellatoidea*, 1 *C. krusei* and 1 *C. rugosa* "Table 1".

In animals, 39 (42.9%) fungal isolates were recovered; of which 36 (39.6%) were mycelial fungi; which were recognized as 13 *Aspergillus niger*, 8 *Aspergillus flavus*, 2 *Aspergillus fumigatus*, 3 *Aspergillus nidulans* 6 *Penicillium* and one of each of *Stachybotrys corda*, *Gliocladium corda*, *Xylohypha bantia* and lower fungi, as well as 3(3.33%) isolates were *C. albicans* "Table 2".

In broiler chickens, 39 (39%) fungal isolates were recovered including 3 mycelial fungi; 2 *A. niger* and 1 *A. flavus* and 36 yeast isolates of which 34 isolates were recognized as *Candida* species; 19 *C. albicans*, 6 *C. pseudotropicalis*, 5 *C. krusei* and 4 *C. rugosa*, while only 2 isolates were recognized as *Cryptococcus* species, *C. neoformans* and *C. laurentii* "Table 3".

Chlamyospore Production and Germ Tube Test.

Among the tested yeasts (49 isolates), all *C. albicans* isolates (29 isolates) were the only producing chlamyospores and forming germ tube while the rest of *Candida* species as well as *Cryptococcus* species did not Fig. 1 and 2.

Polymerase Chain Reaction

All the 3 isolates confirmed morphologically and biochemically as being *C. albicans* showed positive results with PCR test using Oligonucleotide primer that amplifies a 172 bp fragment in *SAP3* gene of *C. albicans* not Fig. 3.

Antifungal Activity of Essential Oils

Thyme oil at concentrations of 0.25, 0.5 and 1% completely inhibited the growth of all the tested fungal isolates using agar dilution method. Clove and cinnamon oils completely inhibited the growth of all the tested fungal isolates at a concentration of 6% while at concentrations of 2, 3 and 4% the fungal growth occurred but with gradual retardation with increased concentration where the fungal growth appeared at the 2nd, 3rd and 4th days, respectively. On the other hand, concentrations of 0.5 and 1% have no effect on the fungal growth Table 4 and Fig. 4-6.

Discussion

Fungi especially yeasts are found on a wide variety of substances such as soil, plants, water, nectar of flowers, fruits, trees and exudates of animals. They cause diseases in both man and animals such as thrush, disseminated candidosis, cryptococcosis and mastitis (Asfour *et al.*, 2009). The reproductive tracts of different animals are the major reservoir of yeasts such as *C. albicans* and *C. neoformans* (El-Naggar *et al.*, 1999). Moreover, the genera of *Aspergillus* and *Penicillium* can grow in a suitable substrate under appropriate conditions. In poultry, the fungal diseases have become problematic as bacterial and viral diseases (Darwish, 1989). Moniliasis or crop Mycosis is a disease that primarily affects the upper digestive tract of all birds and is characterized by whitish thickened areas of the crop and proventriculus, erosions in the gizzard and inflammation of the vent area.

Belonging the prevalence of fungal infection in human, "Table 1" showed that the recovery rate of fungal isolates was 61.1% including 1 *A. flavus* (5.6%) and 10 *Candida* species (55.5%) which were identified morphologically, biochemically and biologically as 7 *C. albicans*, 1 *C. stellatoidea*, 1 *C. krusei* and 1 *C. rugosa*. Such results were higher than those of Bauters *et al.* (2002) who conducted a study at Ghent University Hospital, 612 patients (237 post-menopausal) participated. The participants included both those who had complaints of vaginitis and those who did not. This study showed that yeast was present in 20.1% of the overall sample and 18.6% of the post-menopausal women. Also, Sobel (2005) found that 20% of healthy asymptomatic women, *Candida* species can be found in the lower genital tract flora. On the other hand, these results were lower than those of Odds (1988) who recovered *C. albicans* from 85-90% of all cases of vulvovaginal candidiasis, followed by *C. glabrata* (5-10%), *C. tropicalis* (3-5%) and other species. Also, Beigi *et al.* (2004) studied 1248 asymptomatic (18-30 years old) women and found that yeast was present in 70% of the women at least once and in 30% of the women during all four visits over a one year period.

According to the results illustrated in “Table 2”, the prevalence rate of fungal infection in animals was 42.9% where 36 (39.6%) of them were mycelial fungi including 13 *Aspergillus niger*, 8 *A. flavus*, 2 *A. fumigatus*, 3 *A. nidulans* 6 *Penicillium* and one of each of *Stachybotrys corda*, *Gliocladium corda*, *Xylohypha bantia* and lower fungi, while 3 (3.3%) were *C. albicans*. These results run hand to hand with those of Abou-Elmagd *et al.* (2011) who recovered yeasts from the reproductive tract of apparently healthy buffaloes, cattle and sheep in percentages of 3.33, 4.54 and 1.81%, respectively. This finding might have a good support from the speculation that the opportunistic yeasts under many stress factors could become potential pathogenic that establish a disease condition or may be introduced to the vagina on top of the secondary infections.

These results were lower than those of Misra *et al.* (1984) who yielded fungi from 80% of cultures of fetal organs and placenta from cows which had aborted, while fungi were recovered from 66.6% of uterine swabs from infertile cows. Meanwhile, these results were higher than those of Dafalla *et al.* (1984) who isolated filamentous fungi and yeasts from 19 cows with a prevalence rate of 14.4%. Thirteen of the 19 cows were repeat breeders, while abortion had occurred in two of those reported to be fertile. Giri *et al.* (1994) where prevalence of fungal infection in repeat breeder cattle and buffaloes was 6% and also they were greater than that of Kolev *et al.* (1983) where molds represented 2.54% of the isolated organisms from the investigated cows that exhibited disturbances in their reproduction and had abortions, or gave birth to calves that were affected with diseases or died according to which was very different from our results may be because we isolated from both apparently normal animals and from animals with reproductive disorders while they isolated from animals with reproductive disturbances only. This may suggest that the samples of the present study were taken from area of low hygienic measures. Moreover, Krogh (1985) in Denmark reported that the incidence of mycotic abortion in cattle was 14%. Half of the cases occurred during the three months, December-February (the same conditions of isolation in the present study).

Concerning the fungal species recovered from vagina of cattle and buffaloes, the present results agreed with those of Dafalla *et al.* (1984) who found that the commonest isolates were *Aspergillus flavus*, *A. fumigatus*, *A. niger* and *Penicillium* species. Also, Ainsworth and Austwick (1973) reported that *Aspergillus* species, *Penicillium* species and yeast could cause genital disorder; in cows and buffaloes. Moreover, Misra *et al.* (1984) recovered 88 isolates; from cattle and buffaloes, mostly belonged to the genera *Aspergillus*, *Candida* and *Penicillium* and also Garoussi *et al.* (2007) observed the occurrence of fungi (mainly *Penicillium* and yeast) in cervicovaginal fluids of Holstein dairy cows with

or without reproduction diseases and suggested that those microorganisms are members of the resident or transitory of cervicovaginal flora of Holstein dairy cows.

In the present study, the prevalence rate of fungal isolation from broiler chickens was 39% including 3% mycelial fungi; 2% *A. niger* and 1% *A. flavus* and 36% yeast of which 34% were *Candida* species; 19% *C. albicans*, 6% *C. pseudotropicalis*, 5% *C. krusei* and 4% *C. rugosa*, while only 2% of isolates were recognized as *Cryptococcus* species, 1% *C. neoformans* and % *C. laurentii* “Table 3”. Such results were nearly similar to those of Wyatt and Hamilton (1975) where the mean incidence of *Candida* in the crops was 32.3%. They studied the crops from four field outbreaks of crop mycosis. Three of the four cases of crop mycosis were characterized by multiple strains of *C. albicans* in the crop. In one case, *C. parapsilosis* also was isolated from the crop. They also found that less than 1% exhibited visible lesions attributable to *Candida*. *C. albicans* comprised 95% of the isolates while *C. ravautii*, *C. salmonicola*, *C. guilliermondi*, *C. papapsilosis*, *C. catenulata* and *C. brumptii* comprised the remainder. The population of *Candida* in the crops of birds found positive was of low magnitude in the majority of the chickens examined. On the other hand, these results were higher than those of Pennycott *et al.* (2003) who isolated *C. albicans* from chicken's samples in a percentage of (12.15%).

In the present study, *C. albicans* was the most prevalent fungal isolate in animal, human and chickens. This result was in agreement with Wilson and Plunkett (1970) who reported that *C. albicans* is the primary causative agent of candidiasis, the most common form of mycotic infection. They added that it can be found to naturally colonize the skin, buccal mucosa, intestinal tract and vagina mucosa. Also our results run hand to hand with those of Chengappa *et al.* (1984) who mentioned that *Candida* species are opportunistic fungi, occurring as normal inhabitants of the digestive tract, oral cavity and vagina of humans and many of our domestic animals. They also found that *C. albicans*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* represented 71% of the *Candida* species isolated from domestic animals. Moreover, Foley and Schlafer (1987); in a retrospective study on yeast-associated bovine abortion, recovered *Candida albicans*, *Candida krusei*, *Candida tropicalis* and *C. parapsilosis* from placental lesions as well as from the lung, liver, intestines, abomasum and heart.

Chlamydospore production is considered the most important diagnostic feature in the laboratory identification of *Candida albicans* (Benham, 1931). Corn meal infusion agar has been most satisfactory for this purpose but certain inconveniences in the preparation of this medium led Taschdjian (1953) to develop a rice extract agar for chlamydospore production. The medium is relatively simple to prepare

from readily available materials and its value in the stimulation of chlamydospore production has been confirmed by Sina and Reiss (1975).

The germ tube test provides a simple, reliable and economical procedure for the presumptive identification of *C. albicans*. About 95% of the clinical isolates produce germ tubes when incubated in serum at 35°C for 2.5 to 3 h. A germ tube represents the initiation of a hypha directly from the yeast cell. They have parallel walls at their point of origin. Germ tube formation is influenced by the medium, inoculum size and temperature of incubation. Fresh normal pooled human sera or a commercially available germ tube solution (Remel Lenexa kansa) are to be used as the medium for the test. The inoculum should result in a very faintly turbid serum suspension. Over-inoculation will inhibit the development of germ tubes (Alexopolous *et al.*, 1996).

The present results showed that, *C. albicans* isolates (29 isolates) were the only producing chlamydospores and forming germ tube while the rest of *Candida* species as well as *Cryptococcus* species did not and both tests were considered additional confirming laboratory tests for the presumptive identification.

C. albicans is one of the most medically important fungi because of its high frequency as a commensal and pathogenic microorganism, strain typing and delineation of the species are essential for understanding its biology, epidemiology and population structure. Recent data indicated that, molecular techniques based on PCR and RAPD-PCR have been used as tool for diagnosis of several fungal species (Noumi *et al.*, 2009). PCR and RAPD-PCR patterns enabled the direct identification of common opportunistic pathogenic *Candida* species, including *C. albicans* (Muñoz *et al.*, 2003)

In the present study, the PCR test was applied; as a confirmation, on randomly selected 3 isolates which were morphologically and biochemically identified as *C. albicans* (one of animal origin, one of human origin and one of chicken origin). The results shown in "Photo. 3" revealed that all the 3 isolates confirmed morphologically, biochemically and biologically as being *C. albicans* showed positive results with PCR test using Oligonucleotide primer that amplifies a 172 bp fragment in *SAP3* gene of *C. albicans*.

The main advantage of using amplification targets from regions of DNA which are conserved among all *Candida* species is that a PCR product can be obtained from all species using a single set of PCR primers and conditions optimal for that set of primers (Coignard *et al.*, 2004). Following amplification, species-specific probes can be designed from the more variable DNA regions, located between primer binding sites, for the identification of specific organisms.

The excessive uses of antibiotics, corticosteroids, immunosuppressive drugs as well as chronic diseases are

the major contributing factors in increasing the incidence of fungal diseases (Das and Josef, 2005).

The uses of plant-derived products as disease control agents have been studied, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Lee *et al.*, 2007). Therefore, there has been increasing interest to replace synthetic preservatives with natural, effective and nontoxic compounds. Those are; in the first place, extracts and essential oils of spices and herbs (Smid and Gorris, 1999).

Previous studies have reported antifungal activity for clove oil and eugenol against yeasts and filamentous fungi, such as several food-borne fungal species (López *et al.*, 2007), human pathogenic fungi (Chaieb *et al.*, 2007) as well as animal pathogens (Ahmad *et al.*, 2005). Clinical experience showed that the efficacy of antimicrobial agents depends not only on their direct effect on a given microorganism but also on the functional activity of the host immune system (Tullio *et al.*, 2012).

In the present study, the antifungal activities of essential oils (thyme, clove and cinnamon) using agar dilution method were estimated Table 4 and Fig. 4-6. Thyme oil at concentrations of 0.25, 0.5 and 1% completely inhibited the growth of all the tested fungal isolates. Clove and cinnamon oils completely inhibited the growth of fungal isolates at a concentration of 6% while at concentrations of 2, 3 and 4% the fungal growth occurred but with gradual retardation with increased concentration where the fungal growth appeared after the 2nd, 3rd and 4th days, respectively. On the other hand, concentrations of 0.5 and 1% have no effect on the fungal growth.

Concerning thyme oil, our results were supported with that of Elgayyar *et al.* (2001) who reported that thyme EO is apparently among the best inhibitors of yeast activity and Pozzatti *et al.* (2008) who reported the antifungal activity of thyme and other essential oils. Also, Gutiérrez *et al.* (2010) reported that the antimicrobial efficiency of thymol and carvacrol against *C. albicans* and *A. flavus* showed complete inhibition of these microorganisms. Moreover, Pagiotti *et al.* (2011) reported that Thyme EO showed antifungal activity against all of the tested fungal isolates and fungal growth inhibition by essential oil was accompanied by marked morphological and cytological changes.

Thyme EO has high contents of carvacrol, thymol which displayed very low minimum inhibitory concentrations and minimum fungicidal concentrations against yeasts and molds. They also inhibited the germ tube formation at sub-inhibitory concentrations in *C. albicans*. The antifungal effect of thyme may be interpreted by reduction of ergosterol content; the major sterol component in fungal cell membrane, (Pinto *et al.*, 2006). Therefore, thyme and its main components were

found to display a broad fungicidal activity through the disruption of cytoplasmic membrane integrity leading to leakage of vital intracellular compounds (Vale-Silva *et al.*, 2010). Moreover, thyme led to rapid metabolic arrest, disruption of the plasma membrane and consequently cell death. Also, Braga and Ricci (2011) interpreted the antifungal effect of thymol against *C. albicans* due to interfering with the envelope. They observed that cell showed major morphostructural deformities with envelope damage becoming greater at increasing thymol concentrations and longer times of incubation, including the number of flattened cells with surface folds, cells with holes and collapsed cells and ghosts. Thymol is an amphipathic monoterpene, which was suggested to affect cell membrane structure by generating asymmetries and membrane tensions. This is confirmed by the fact that terpenes alter cell permeability by entering between the fatty acyl chains making up the membrane lipid bilayers, disrupting lipid packing and changing membrane fluidity. All of these phenomena lead to major surface alterations and deformities that also reduce the ability of fungi to adhere to mucosal cells and decrease their virulence and infectiousness. Moreover, the thyme EO at sub-MIC/MIC concentrations significantly enhancing the intracellular killing activity by polymorphonuclear granulocytes against *C. albicans* (Tullio *et al.*, 2012).

Belonging clove oil, our results were supported with that of Ahmad *et al.* (2005) who evaluated the antifungal activity of clove oil which was found to possess strong antifungal activity against opportunistic fungal pathogens such as *C. albicans*, *C. neoformans* and *A. fumigatus*. The oil was found to be extremely successful in the treatment of experimental murine vaginitis in model animals. On evaluating various formulations, topical administration of the liposomized clove oil was found to be most effective against treatment of vaginal candidiasis. Also, Chaieb *et al.* (2007) cleared that clove oil showed a powerful antifungal activity; and it can be used as an easily accessible source of natural antioxidants and in pharmaceutical applications. Moreover, Pinto *et al.* (2009) indicated that clove oil and eugenol have considerable antifungal activity against clinically relevant fungi, including fluconazole-resistant strains, deserving further investigation for clinical application in the treatment of fungal infections.

Pinto *et al.* (2009) explained the antifungal effect of clove EO due to ergosterol by eugenol; the major phenolic component of clove essential oil, which caused a considerable reduction in the quantity of ergosterol. Germ tube formation by *C. albicans* was completely or almost completely inhibited by clove oil and eugenol concentrations below the MIC values. Also, He *et al.* (2007) investigated the potent antifungal activity eugenol and their effects on preformed biofilms, adherent cells,

subsequent biofilm formation and cell morphogenesis of *C. albicans*. The results indicated that the effect of eugenol on adherent cells and subsequent biofilm formation was dependent on the initial adherence time and the concentration of this compound and that eugenol can inhibit germ tube formation of *C. albicans* cells. In addition, using human erythrocytes, eugenol showed low hemolytic activity. These results indicated that eugenol displayed potent activity against *C. albicans* biofilms *in vitro* with low cytotoxicity and therefore has potential therapeutic implication for biofilm-associated candidal infections.

In case of cinnamon oil, our results were supported with that of López *et al.* (2007) who recorded a complete growth inhibition of the fungi by cinnamon EO. and Carmo *et al.* (2008) who concluded that cinnamon EO could be known as potential antifungal compound, particularly, to protect against the growth of *Aspergillus* species and also with Gutiérrez *et al.* (2010) who reported that the antimicrobial efficiency of cinnamon against *C. albicans* and *A. flavus* showed complete inhibition of these microorganisms. Moreover, Pires *et al.* (2011) and Wang *et al.* (2012) reported the strong antimicrobial action of cinnamon oil for different pathogenic *Candida* species.

We suggested that the antifungal activity of cinnamon EO may be due to disruption in the fungal wall and this suggestion was in agreement with that reported by Carmo *et al.* (2008) who found that cinnamon oil strongly inhibited the radial mycelial growth of *Aspergillus* species and associated with morphological changes as decreased conidiation, leakage of cytoplasm, loss of pigmentation and disrupted cell structure indicating fungal wall degeneration.

Collectively, these results run hand to hand with those of Suhr and Nielsen (2003) who reported that thyme, clove and cinnamon to be the overall best long-term inhibitors upon direct addition to media. These results also were supported by many authors; Pawar and Thaker (2006) who worked on cinnamon and clove reported their effects on *Aspergillus* species by reduction of spore formation and Omidbeygi *et al.* (2007) who evaluated the antifungal activity of thyme, summer savory and clove in culture medium. Also, Braga *et al.* (2007) tested the anticandidal activity of eugenol and thymol alone or in combination against *C. albicans*. Certain combinations of the two molecules led to a synergistic effect, which is interesting in the view of potentiating their inhibition of *C. albicans* colonization and infectiousness. Moreover, Gutiérrez *et al.* (2010) reported that the antimicrobial efficiency of cinnamon and thyme and their chemical descriptors (cinnamaldehyde, thymol and carvacrol) against yeast (*C. albicans*) and mold (*A. flavus*).

According to these findings, among the tested essential oils, thyme has the highest antifungal activity as it inhibited the growth of fungi completely at all concentrations (0.25, 0.5 and 1%) while in case of clove and cinnamon, the complete fungal growth inhibition occurred at concentration of 6% while the lower concentrations (1-4%) were not accompanied with growth inhibition. These results agree with that of Omidbeygi *et al.* (2007) who found that thyme has the highest antifungal activity, followed by summer savory and clove EOs. On the other, these results disagree with those of Pires *et al.* (2011) who investigated the anticandidal activity of 16 essential oils and found that cinnamon was the most active essential oil showing anticandidal activity. And those of López *et al.* (2007) who reported cinnamon EO completely inhibited the growth of the fungi either molds or yeasts using a nominal concentration of 4% (w/w) of fortified cinnamon.

Conclusion

Also, in this study, it was revealed that increasing the antifungal activity of the oils was enhanced by increasing their concentrations. This finding agreed with the report of Sharma and Tripath (2008) who reported that higher concentration of antimicrobial substance showed appreciation in growth inhibition.

It was concluded that thyme, clove and cinnamon EOs; especially thyme and their phenolic compounds have a strong antifungal activities against molds and yeasts. Therefore, they may have potential for use in the development of clinically useful antifungal preparations.

Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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