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Full Length Research Paper

Comparative studies of antimycotic potential of thyme and clove oil extracts with antifungal antibiotics on *Candida albicans*

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This study was undertaken to compare the antifungal properties of clove (*Syzygium aromaticus*) and thyme (*Thymus vulgaris*) oils with the synthetic antifungal agents notably, amphotericin B, itraconazole, fluconazole and ketoconazole against *Candida albicans in vitro*. This is necessary if oil extracts can replace antifungal antibiotics as agents for the treatment of candidiasis. The minimal inhibitory concentrations (MICs) of the essential oils were determined by first solubilizing them with dimethyl sulphoxide (DMSO) followed by serial two-fold dilutions in Sabouraud's broth using *Candida albicans* (ATCC 10231) control and other 10 isolates of *C. albicans*. The minimum inhibitory concentrations (MICs) of the oils, showing no visible fungal growth, varied from 1.0 x 10^3 to 2.5 x 10^3 µg/mL for clove and 4.6 x 10^2 to 9.3 x 10^2 µg/mL for thyme while their minimum antifungal end-points were 2.5 x 10^3 and 1.9 x 10^3µg/mL, respectively. Thyme oil was more antifungal than clove oil. The mean of the MIC's of the antifungal agents notably amphotericin B, ketoconazole, fluconazole and itraconazole were 0.031, 0.015, 1.9, and 0.168 µg/mL, respectively, indicating that they are more antifungal than the oil extracts. The experiment indicates that, *in vitro*, the antifungal antibiotics are more antifungal than the essential oils. Perhaps, the mode of extraction of the oils may have contributed to the active agents being suboptimal in the extracts.

Key words: *Candida* spp., thyme, clove, antifungal antibiotics, essential oil.

INTRODUCTION

Many plant extracts called essential oils possess antimicrobial properties and some of them are used as antiseptics, antispasmodic preparations or as food preservatives and enhancers of food flavours (Panizzi et al., 1993; Arora and Kaur, 1999). It is postulated that the importance of the essential oils to plants is to act as phytoprotective agents defending the tree from herbivores and pathogenic attack (Gijzen et al., 1991).

Studies have shown that thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticum*) are antimycotic and suggestions have been made that their oil extracts be incorporated into some pharmaceutical preparations as preservatives especially for cosmetic products and as a drug for treatment of *Candida* infections (Manou et al., 1998; Arras and Usai, 2001). The essential ingredients responsible for their antifungal activities are thymol and carvacrol for thyme and eugenol for clove (Panizzi et al., 1993).

Though their mode of killing microbes are not extensively documented, Pina-Vaz et al. (2004) showed that thyme oil produced lesions in the plasma membrane of fungi thereby allowing the organism to die by the efflux of the contents of the plasma membrane.

Incidence of oral, cutaneous or vulvovaginal candidiasis following impaired immunity such as in diabetes mellitus, human immunodeficiency virus infection, cancer, leukaemia, transplants or in the prolonged use of broad spectrum antibiotics, is usually treated with antifungal drugs (Terrell and Hughes, 1992; Keele et al., 2001). However, most of the *C. albicans* strains have become resistant to the antibiotics (Gibbons, 1992; Laguma et

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al, 1994; Dupont et al., 1996). In some instances, the uses of such synthetic antifungal drugs have resulted in nephrotoxicity or hepatotoxicity in the patients receiving them (Dupont et al., 1996; Kauffman and Carver, 1997). The search therefore for agents that can eliminate the side effects of these antibiotics and at the same time control or eliminate the emergence of resistant strains of *C. albicans* is needed. In this regard, the use of essential oils of plant calls to mind since the use of herbs in Indian and Chinese folk medicine has proved effective and is well documented (Gibbons, 1992; Mehmood et al., 1999). Moreover, some authors (Chami et al., 2004; Ahmad et al., 2005) claimed that these herbs could replace the use of synthetic antifungals in the treatment of candidiasis.

The present study, therefore, aims at comparing, *in vitro*, the minimum inhibitory concentration (MIC) of clove and thyme essential oils with the MIC of some antibiotics used in the treatment of candidiasis. The study also aims to verify if oil extracts can be recommended or incurporated in some preparations like suppositories or gels used for the treatment of such infections like vaginal thrush, oral and cutaneous candidiasis. Presently, only antifungal synthetic antibiotics are incorporated in such preparations.

MATERIALS AND METHODS

Sources of thyme and Clove

Thyme (*Thymus vulgaris*) used in this experiment came from Oman while clove (*Syzygium aromaticum*) was from Zanzibar. Their leaves, stems, buds and flowers were used after identification of plants' species by horticulturists.

Extraction of essential oils

A locally fabricated distiller comprising of Stove Still™ (Essential Oil University, New Albany, Indiana) connected to a glass steam condenser through a plastic hose was used. 30 g of each herb was in turn fed into the Still and 120 ml of distilled water was added. Heating was at 100°C at standard pressure. The volatile vapours that condensed at water temperature of 8°C were collected in glass bottles, labeled and called essential oils. They were stored in the fridge till ready for use.

Screening for antifugal activity

Each essential oil was diluted initially 1:2 in dimethyl sulphoxide (DMSO) to solubilize. This was followed by doubling dilution from 1:2 to 1:16 in sterile saline. An overnight growth of *C. albicans* (ATCC 10231) on Sabouraud dextrose agar (SDA) was purified and identified using germ tube, API 20 AUX (Biomereux, France) and Candifast (International Microbiol. France) techniques. Sabouraud dextrose agar (SDA, Oxoid, England) plates were swabbed with 10^5 colony forming units per milliliter (CFU /mL) of the control organism determined with Mcfarland's Standard Opacity tube 0.5. Holes of 4 mm were made with disposable tips and plugs were removed with sterile needles. 50 µl of the diluted oils were put into the wells using sterile pipettes. The plates were subsequently incubated at 37°C for 48 h. Zones or no zones of growth inhibition were recorded.

Determination of minimum inhibitory concentrations (MICs)

Briefly, 1 ml of thyme or clove extracts when weighed gave 0.96 and 1.06 g respectively. 500 µl of each were solubilised in glass bottles with 500 µl of DMSO at room temperature for 15 min. Thereafter, doubling dilutions in duplicates, from 1:4 to 1: 8192, equivalent to 1.1×10^2 - 2.4×10^5 µg/mL and 1.3×10^2 - 2.6×10^5 µg/mL respectively, were made in Sabouraud broth (SAB). *C. albicans* (ATCC 10231) and 10 other isolates of *C. albicans* each at 10^5 CFU/mL were separately added to the dilutions using 0.5 mL volumes (Tables 1 and 2). Controls comprising of 0.5 ml of DMSO with 0.5 mL of ATCC (DMSO control), 0.5 mL of each oil with 0.5 mL of SAB (oil control), 0.5 mL of ATCC 10231 with 0.5 mL of SAB (ATCC control) and 0.5 mL of each isolate with 0.5 mL of SAB (isolate control) were made and incubated with the diluted oils at 37°C for 48 h. The MIC of each oil was defined as the minimum concentration showing no visible growth.

Minimum fungicidal concentration (MFC)

It was discovered at the MIC readings that turbidity appeared at high concentrations of the oils at 3.0×10^4 – 2.4×10^5 µg/mL (thyme) and 3.3×10^4 – 2.6×10^5 µg/mL (clove) but cleared at lower concentrations. To determine the exact MIC/MFC of the oils, each dilution was plated on Sabouraud dextrose agar, incubated for 48 h at 37°C. The lowest concentration showing growth inhibition was taken as the end point (MFC).

Determination of the MIC of antifungal antibiotics

The antifungal agents tested were ketoconazole, itracona-zole, amphotericin B and fluconazole. The control organism, ATCC 10231 and the test organisms (*C. albicans*) were standardized as for the MIC determination using Mcfarland's opacity 0.5 tubes at 10^5 CFU/mL. Our inability to obtain pure liquid concentrate of the antifungal antibiotics gave raise to our using for this experiment the E-test strips which were readily available in our laboratory. The test was put up according to the manufacturer's instruction (AB Biodisk, Sweden (1993)). Briefly, sterile non-toxic swabs dipped separately into the control and test orga-nisms were used to swab dried SDA medium. The plates were left for 10 min at room temperature to allow excess moisture to absorb into the agar. With a pair of sterile forceps, each strip containing each antibiotic was separately placed on the plates so that the MIC scale for each antibiotic was facing upwards and the whole length of the strips was in complete contact with the agar surface. The plates were incubated at 37°C for 24 h. The reading of the MICs took three modes. It was read at the point where the zones of inhibition intersected the MIC scale on the strip. It was also reported as greater than the highest value on the reading scale if no inhibition ellipse was seen or less than the lowest value on the lowest concentration of antibiotics in the strip.

RESULTS

MICs of the essential oils and antifungal agents

Figures 1a and 1b show the micrographs of thyme and clove used in the experiment. Both thyme and clove essential oils showed growth inhibitions of more than 25 mm (diameter) up to 1:16 dilution. Tables 1 and 2 show the end points of dilution of the oils and their antifungal end-points. While thyme oil had MIC of 4.6×10^2 µg/mL with ATCC 10231 and three isolates of *C. albicans*, se-

A B

Figure 1. A: Clove from Zanzibar, B: Omani thyme.

ven isolates had 9.3 x 10^2 µg/mL (Table 1). In the case of clove, both the control organism (ATCC 10231) and eight isolates had MIC of 1.0 x 10^3 µg/mL respectively while the MIC of two isolates were 2.5 x 10^3 µg/mL (Table 2).

The minimum fungicidal concentration (MFC) of thyme and clove for all the organisms were 1.9 x 10^3 µg/mL for thyme and 2.5 x 10^3 µg/mL for clove (Tables 3 and 4).

The MICs of the antifungal antibiotics for both the control and the isolates are represented in Table 5 while Figure 2 is their histogram. Their mean values vary from 0.002-1.9µg/mL with amphotericin B having lowest MIC (0.002µg/mL) and fluconazole the highest (1.9µg/mL). Figure 3 represents the micrograph and intersections of growth of the organisms with the strips of different antibiotics.

Comparative values of essential oils with the antifungal antibiotics

Table 6 gives a summary of the comparison between the antifungal activities of essential oils with the antifungal antibiotics. Whereas clove and thyme oils had MICs mean values of 1300 and 786 µg/mL, respectively, with the test organisms, amphotericin B, ketoconazole, fluconazole and itraconazole were 0.031, 0.015, 1.90 and 0.168 µg/mL, respectively.

DISCUSSION

Thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticcum*) essential oils are known to possess antimicrobial activities against a wide range of microorganisms including fungi (Hammer et al., 1999; Cosentino et al., 1999; Dorman and Deans, 2000; Arras and Usai, 2001). However, their antimicrobial activities were found higher against fungi than against bacteria (Hammer et al., 1999). Their antimicrobial activities have made it possible to use them as preservatives in foods and in folk medicine (Panizzi et al., 1993; Mehmood et al., 1999, Cosentino et al., 1999).

Though the modes of action of the essential oils are being debated, their antimicrobial agents include thymol and carvacrol found in thyme and eugenol found in clove. These substances acting alone or in combination with other agents present in the extracts may result in broad spectrum of antifungal or antibacterial activities currently observed in these herbs (Hammer et al., 1999; Cosentino et al., 1999; Pina-Vaz, 2004). Pina-Vaz (2004) found carvacrol in clove able to kill *C. albicans* by producing lesions in the plasma membrane. The organism died through efflux of the contents of the plasma membrane.

There are discrepancies in the end-points of the MICs of the essential oils observed by various workers (Hili et al., 1997; Hammer et al., 1999; Pina-Vaz et al., 2004). In our experiment, thyme extract had an MIC between 4.6 - 9.3 x 10^2 µg/mL and MFC of 1.9 x 10^3 µg/mL while clove was between 1.0 - 2.5 x 10^3 µg/mL and had MFC of 2.5 x 10^3 µg/mL (Tables 1, 2, 3 and 4). The differences in the MICs may be due to the chemical composition and mode of extraction of the extracts which Sing-Sangwan et al. (1994) and Senatore (1996) found to affect the antifungal or antibacterial potential of the oils.

The essential oils used in our experiment were extracted using the equipment and methods at our disposal and the oils were found to contain no molecules of water. Besides the chemical composition which influences the antifungal behavior of the oil extracts, Sing-Sangwan et al. (1994) found that the origin of the herbs, the season of the year they were harvested, their moisture content at harvest and post-harvest storage affect their chemical composition. Ahmad et al. (2005)

Table 1. Minimum inhibitory concentration (MIC) of thyme oil.

C. albicans	Dilutions											
	1/ 4	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**	**1/4096**	**1/8192**
Control	-	-	-	-	-	-	-	-	-	-	+	+
1	-	-	-	-	-	-	-	-	-	+	+	+
2	-	-	-	-	-	-	-	-	-	-	+	+
3	-	-	-	-	-	-	-	-	-	-	+	+
4	-	-	-	-	-	-	-	-	-	+	+	+
5	-	-	-	-	-	-	-	-	-	+	+	+
6	-	-	-	-	-	-	-	-	-	-	+	+
7	-	-	-	-	-	-	-	-	-	+	+	+
8	-	-	-	-	-	-	-	-	-	+	+	+
9	-	-	-	-	-	-	-	-	-	+	+	+
10	-	-	-	-	-	-	-	-	-	+	+	+
µg/mL	$2.4x10^5$	$1.2x10^5$	$5.9x10^4$	$3.0x10^4$	$1.5x10^4$	$7.4x10^3$	$3.7x10^3$	$1.9x10^3$	$9.3x10^2$	$4.6x10^2$	$2.3x10^2$	$1.1x10^2$

Table 2. Minimum inhibitory concentration of clove oil.

	Dilutions											
C. albicans	**1/ 4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**	**1/4096**	**1/8192**
Control (ATCC10231)	-	-	-	-	-	-	-	-	-	+	+	+
1	-	-	-	-	-	-	-	-	-	+	+	+
2	-	-	-	-	-	-	-	-	-	+	+	+
3	-	-	-	-	-	-	-	-	-	+	+	+
4	-	-	-	-	-	-	-	-	+	+	+	+
5	-	-	-	-	-	-	-	-	-	+	+	+
6	-	-	-	-	-	-	-	-	-	+	+	+
7	-	-	-	-	-	-	-	-	+	+	+	+
8	-	-	-	-	-	-	-	-	-	+	+	+
9	-	-	-	-	-	-	-	-	-	+	+	+
10	-	-	-	-	-	-	-	-	-	+	+	+
µg/mL	2.6×10^5	1.3×10^5	6.6×10^4	3.3×10^4	1.6×10^4	8.2×10^3	4.1×10^3	2.5×10^3	1.0×10^3	5.2×10^2	2.6×10^2	1.3×10^2

Tabe 3. Minimum fungicidal concentration (MFC) of thyme oil.

	Dilutions											
C. albicans	**1/ 4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**	**1/4096**	**1/8192**
Control (ATCC10231)	-	-	-	-	-	-	-	-	+	+	+	+
1	-	-	-	-	-	-	-	-	+	+	+	+
2	-	-	-	-	-	-	-	-	+	+	+	+
3	-	-	-	-	-	-	-	-	+	+	+	+
4	-	-	-	-	-	-	-	-	+	+	+	+
5	-	-	-	-	-	-	-	-	+	+	+	+
6	-	-	-	-	-	-	-	-	+	-	+	+
7	-	-	-	-	-	-	-	-	+	+	+	+
8	-	-	-	-	-	-	-	-	+	+	+	+
9	-	-	-	-	-	-	-	-	+	+	+	+
10	-	-	-	-	-	-	-	-	+	+	+	+
µg/mL	2.4×10^5	1.2×10^5	5.9×10^4	3.0×10^4	1.5×10^4	7.4×10^3	3.7×10^3	1.9×10^3	9.3×10^2	4.6×10^2	2.3×10^2	1.1×10^2

Table 4. Minimum fungicidal concentration (MFC) of clove oil.

	Dilutions											
C. albicans	**1/ 4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**	**1/4096**	**1/8192**
Control (ATCC10231)	-	-	-	-	-	-	-	-	+	+	+	+
1	-	-	-	-	-	-	-	-	+	+	+	+
2	-	-	-	-	-	-	-	-	+	+	+	+
3	-	-	-	-	-	-	-	-	+	+	+	+
4	-	-	-	-	-	-	-	-	+	+	+	+
5	-	-	-	-	-	-	-	-	+	+	+	+
6	-	-	-	-	-	-	-	-	+	+	+	+
7	-	-	-	-	-	-	-	-	+	+	+	+
8	-	-	-	-	-	-	-	-	+	+	+	+
9	-	-	-	-	-	-	-	-	+	+	+	+
10	-	-	-	-	-	-	-	-	+	+	+	+
µg/mL	2.6×10^5	1.3×10^5	6.6×10^4	3.3×10^4	1.6×10^4	8.2×10^3	4.1×10^3	2.5×10^3	1.0×10^3	5.2×10^2	2.6×10^2	1.3×10^2

Table 5. Minimum inhibitory concentration of antifungal antibiotics by E-test.

Organism	Antifungal agents			
	Amphotericin B	**Ketoconazole**	**Fluconazole**	**Itraconazole**
C.albicans ATCC 10231	0.002	0.006	0.5	0.047
1	0.032	0.008	0.5	0.125
2	0.094	0.016	3.0	0.38
3	0.032	0.032	1.0	0.25
4	0.032	0.016	3.0	0.125
5	0.016	0.012	1.5	0.064
6	0.002	0.008	3.0	0.125
7	0.032	0.012	1.0	0.047
8	0.032	0.023	4.0	0.25
9	0.002	0.012	1.0	0.125
10	0.032	0.012	1.0	0.19
Mean	0.031	0.015	1.900	0.168
Standard Deviation (SD)	0.0255	0.007	1.220	0.100

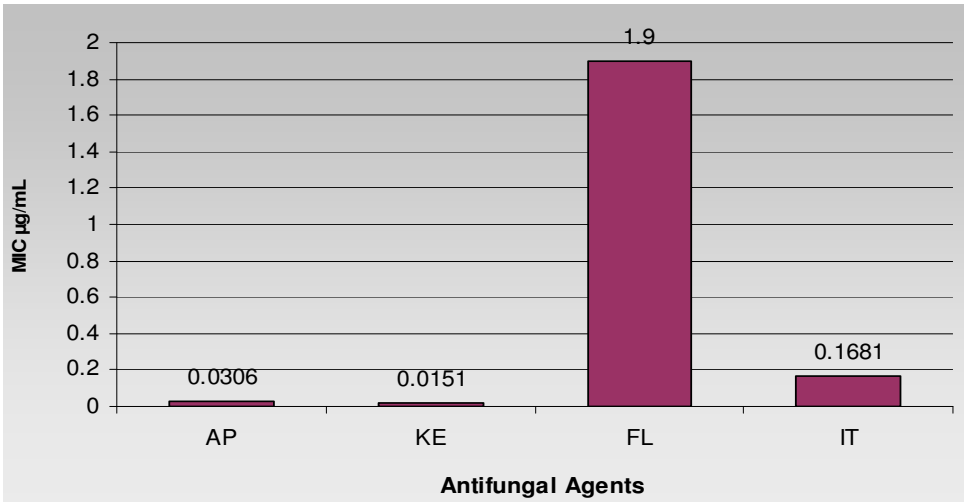

Figure 2. Mean of MIC of antifungal antibiotics by E-test. AP: Amphotericin B, KE**:** Ketoconazole, FL: Fluconazole, IT: Itraconazole

analyzed the eugenol contents of Madagascar and Indian clove extracts and found them to be 89.5 and 72.5% respectively confirming that the country of origin of the plants affects both their chemical composition and their antimicrobial potential. The thyme and clove used in this experiment came from Oman and Zanzibar, respectively, but their post-harvest storage, moisture content and time of harvest were not known even though we found the thyme to possess more antifungal activity than clove.

Our result indicates that the antifungal antibiotics are more antifungal *in vitro* than the essential oils (Tables 5 and 6). The application of any antimycotic agent in clinical medicine entails that any antimycotic agent, *in vitro,* with low MIC with any fungi, indicates susceptibility while high MIC means resistance depending on the acceptable break points of the organism to the antimycotic agent (Miles and Amyes, 1996).

Though Chami et al. (2004) and Ahmad et al. (2005) found that their extracts with high MICs eliminated oral candidiasis in mice faster than nystatin with lower MIC, their experiments were conducted in immunosuppressed mice. Results obtained from such experiments are not always reliable or universally acceptable.

The trailing effect (Figure 3A, B and C) observed in the susceptibility reading of E-test occurred only with antifungal antibiotics (fluconazole, ketoconazole, itraconazole) and not with the extracts or amphotericin B. This indicates that the action of the extracts on the *Candida* species was fungicidal while that of the antibiotics notably, fluconazole, ketoconazole and itraconazole in which the trailing effects were observed was fungistatic. The trailing effect could occur if the incubation temperature of

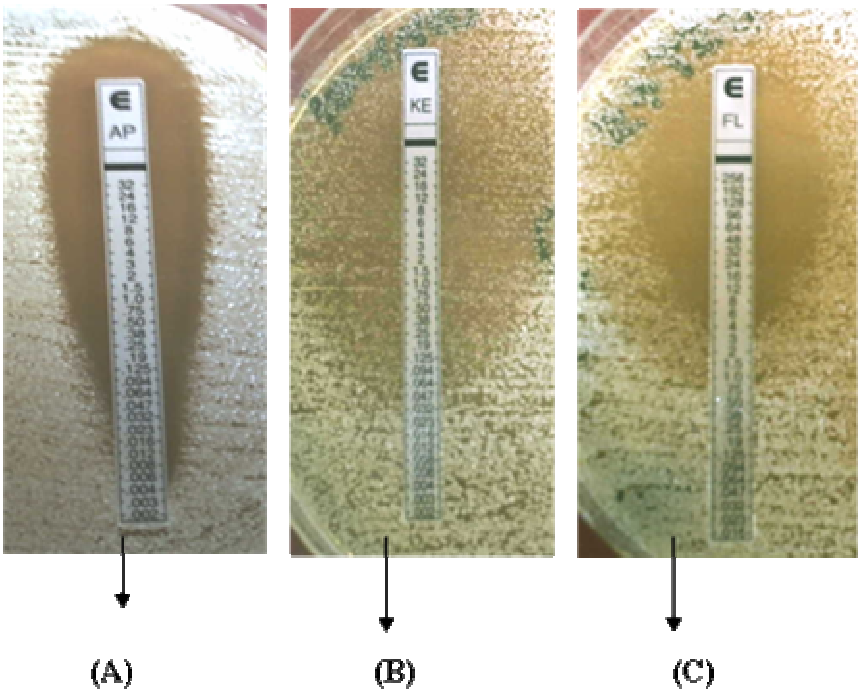

Figure 3. E-test reading patterns. (A) Sharp end point reading. (B) Growth of micro-colonies inside zone of inhibition zone (trailing effect). (C) Double halo, illustrated by the growth of micro-colonies just close to the border of the inhibition zone (trailing effect).

Table 6. Minimal inhibitory concentration (MIC) of clove and thyme extracts compared with E-test of antifungal antibiotics.

Extract	No of organism	MIC (µg/mL)	Mean (µg/mL)	E-test mean (µg/mL)			
clove	8	1.0×10^3	1300	**Amphotericin B**	**Ketoconazole**	**Fluconazole**	**Itraconazole**
	2	2.5×10^3					
Total	10						
thyme	3	4.6×10^2	786				
	7	9.3×10^2		0.031	0.0151	1.900	0.168
Total	10						

the plates (37°C) lowered the antimycotic potential of the antibiotics or if their mode of killing was to arrest the growth of the organisms. Microcolonies of the organisms would then develop within the original zones of growth inhibition once the killing powers of the antibiotics become ineffective.

The turbidity observed at high concentrations but not at lower concentrations of the extracts during the dilutions for MIC and MFC determinations cannot be explained. However, we feel that there may be other ingredients in the extracts insoluble in DMSO used for solubilization of the extracts. Furthermore, the interaction between the extracts, the DMSO and the Sabouraud broth used for the dilution might have resulted in the formation of other hydrophobic molecules. Since these molecules never interfered with our results, their role was not investigated.

Though the search for plant extracts with broad, non-toxic antifungal action is necessary in controlling or elimination of the emergence of resistant strains of *Candida* species, our *in vitro* experiment does not find thyme and clove essentials oils comparable with synthetic antimycotic antibiotics. This may be due to the fact that our extraction method is suboptimal for obtaining high concentrations of active agents in the extracts. It is, therefore, recommend-ed that future investigation will incorporate sequential distillation of mixtures of herbs from different countries to ensure adequate cover for environmental factors which may affect the level of active

ingredients in the extracts.

Conclusion

We have demonstrated that oil extracts of thyme and clove possess antifungal activities. Their antimycotic activity is more with thyme than with clove. *In vitro*, their antifungal properties are lower than the antimycotic synthetic antibiotics used in the treatment of candidiasis. They can, therefore, not be a substitute for antifungal antibiotics currently in use even though some workers recommend them as substitutes based on their work conducted on immunosuppressed mice. However, the search for the effective plant extracts that can be as good as antimycotic synthetic antibiotics should continue since many of them were found to be very useful in therapeutics as well as in clinical medicine.

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