Original article

Comparison of anti-*Candida* activity of thyme, pennyroyal, and lemon essential oils versus antifungal drugs against *Candida* species

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Abstract

Because of resistance and side effects to common antifungal drugs, there have been many studies on the use of herbal antifungal essential oils. In this study, the anti-*Candida* activities of thyme, pennyroyal and lemon essential oils in comparison to antifungal drugs were assessed. The paper disc diffusion method was used to study the inhibitory effects of the essential oils of thyme, pennyroyal and lemon and amphotericin B and fluconazole on different species of *Candida* including *Candida albicans*, *C. krusei* and *C. glabrata*, which are isolated from patients who suffered from vulvovaginal candidiasis, at 25°C and 37°C. The transparent zone around the paper discs showed that thyme essential oil has the greatest effect against different *Candida* species, followed by pennyroyal and lemon. The inhibitory effects of essential oils on the growth of *Candida* at 37°C were better than 25°C. The inhibitory effect of amphotericin B was better than fluconazole. The results of the present study showed that amphotericin B and thyme essential oil had inhibitory effects on the growth of different *Candida* species. Therefore, after performing controlled studies and experimenting with different types of honey formulation, thyme essential oil may be used in treating empirical candidiasis. In conclusion, these essential oils have anti-*Candida* activity, *in vitro* and related to their concentration on paper disc.

Keywords: Anti-*Candida*, Essential oil, Thyme, Pennyroyal, Lemon, Paper disc

Introduction

The resistance of pathogenic fungi, including *Candida albicans* and non-*Candida albicans* species isolated from patients, against antifungal agents has increased [1]. Based on the toxicity and low potency, combined with the increasing side effects of these drugs [2], novel fungal therapies with fewer side effects on humans are urgently required for effective management of candidiasis infections [3,4]. Plants have been used for medicinal purposes since time immemorial [5]. Recently, a number of researchers have reported the antimicrobial effects of various plant extracts against certain pathogens [3]. Medicinal plants have attracted considerable research attention as new sources of antimicrobial agents [6,7]. Wide varieties of plant extracts have antimicrobial and

antimycotic effects and also anti-inflammatory properties [5,8]. Herbal essential oils play a fundamental role in traditional medicine in some countries [9]. Among these, essential oils from natural plant origin have been shown to be active against some pathogenic microorganisms [10]. As it is indicated in the result of some studies that the growth of *C. albicans* can be inhibited by essential oil [6,8,11]. In this matter a number of essential oils such as Lavender, Sage, Clove and Thyme were used [2,8].

The aim of the present study was to assess the anti-*Candida* activities of thyme, pennyroyal and lemon essential oils against some clinical isolates of *Candida* species by using paper disc diffusion to find alternative materials to synthetic antifungal drugs.

Materials and methods

Fungi

Three clinical isolates of *Candida* species, including *C. albicans*, *C. krusei* and *C. glabrata* isolated from women who suffered vulvovaginal candidiasis and also *C. albicans* (PTCC5027) (purchased from institute of industrial and scientific research organization of Iran) were used for sensitivity test. These species were confirmed by common distinction procedures for yeasts. Then the prepared suspensions were kept in 50% glycerin liquid at -70°C. The fungus suspension was cultured on Sabouraud dextrose agar, SDA (Merck, Germany) supplemented with chloramphenicle (SC) and incubated at 30°C for 48h. The yeast was counted by using a Neubauer's chamber and adjusted to 5×10^6 cells/ml.

Essential oils

Three pure herbal essential oils of thyme, pennyroyal, and lemon were purchased from the Barij essential oil pharmaceutical company (Kashan, Iran). These essential oils were kept at 4°C before use and during the process. According to the company instructions, essential oils were emulsified in hexane.

Drugs

Amphotericin B (Sigma Co., USA) and fluconazole (Fuji Co., Japan) were used for positive controls. Amphotericin B and fluconazole were dissolved in 100% Dimethyl Sulfoxid, DMSO (Sigma Co., USA) and distilled water, respectively, to obtain stock solutions at a concentration of 128mg/ml. Small volumes of the stock solutions were stored at -70°C until used. Drugs were diluted in distilled water for final concentration, 8-512mg/ml for amphotericin B and 64-2048mg/ml for fluconazole.

Paper disc diffusion

The paper diffusion assay for sensitivity testing was performed for all essential oils and drugs against *Candida* species using the following procedure: 200µl of the yeast suspension was placed on a surface plate (90mm in diameter) with SC. The yeasts were spread on the surface plate by using 1.8ml of melted agarose with a temperature of 40-50°C. Thirty micro liters of two-fold different dilutions of essential oils and drugs were poured onto the sterile paper discs (6.4mm in diameter) and allowed to dry at room temperature. The impregnated paper discs were placed on the surface of the plates and kept in the incubator at 37°C and 25°C for 48h, separately. The essential oils of thyme, pennyroyal and lemon were used at eight, six and eight dilutions, respectively; and seven and eight dilutions were used for amphotericin B and fluconazole, respectively. Hexane (at the highest concentration similar to the first one, was used for essential oils) and distilled water were used as controls. Each experiment was carried out in duplicate.

Measurement of growth inhibition

Diameter of the transparent zone (*Candida* growth inhibition zone) was measured by a finely calibrated ruler for all the essential

oils and drugs after the incubation period. The mean diameter of the inhibition zone was recorded. The assessment results shown were resistant (lower than 7mm in diameter), dose-dependent (7-11mm in diameter) and sensitive (larger than 11mm in diameter).

Statistical analysis

The statistical method of ANOVA was used for comparison of the effectiveness of the anti-*Candida* activities of essential oils and drugs. P values less than 0.05 were considered significant.

Results

The anti-*Candida* activity of the herbal essential oils showed that thyme essential oil has the most inhibitory effect on different *Candida* species, followed by pennyroyal and lemon. The difference between them was statistically significant ($P<0.001$). Although *C. krusei* was most sensitive to treatment with essential oils, there weren't any significant differences between them. It should be mentioned that the anti-*Candida* effect of essential oils at 37°C was better than 25°C (Table 1). In comparison with control group, 100% and 87.5% of *Candida* species were inhibited by the thyme and pennyroyal essential oils (in dilutions of 6.25-50%), respectively (Fig. 1). Sensitivity of these species to the lemon was about 12.5% (Table 2).

The anti-Candida activity of antifungal drugs

The result of the study of the inhibitory effects of amphotericin B and fluconazole on growth of different species of *Candida* showed that both of them could inhibit *Candida* growth experimentally. The effects at 37°C were better than at 25°C (Table 3). In the same concentration (16-512µg/ml, six concentrations), 70% of the *Candida* species were sensitive to different concentrations of amphotericin B, while this amount for fluconazole was 31% (Table 4 and Fig. 1). However, the diameter of inhibition zone for some concentration of fluconazole was better than amphotericin B. The inhibitory zone around colony made by amphotericin B was less than thyme essential oil but more than pennyroyal.

Discussion

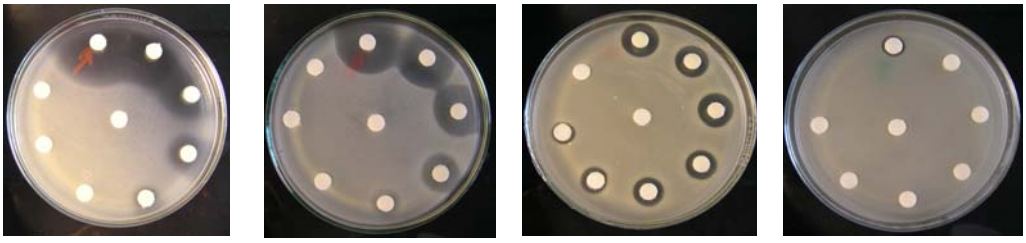
The results of the present study show that thyme essential oil had the most inhibitory effect on *Candida* growth. The effects at 37°C were better than 25°C. Fluconazole had a bigger inhibitory zone, but amphotericin B had a better inhibitory effect on *Candida* species than fluconazole in the same concentration. The anti-*Candida* activities of thyme and pennyroyal essential oils were significantly different, although they were almost similar when compared to lemon.

There are several possible reasons for this: the two come from the same plant family and have similar effective materials; also, diffusion rates on agar plates due to different molecular weight are different. These factors may contribute to variations in the inhibitory zones [3,6]. The better anti-*Candida* activity of thyme has been stated in many reports, and it should also be noted that the anti-*Candida* activity of the thyme essential oil with 50% concentration, used in this study, was similar to other studies [1,4,12].

Table 1: The anti-*Candida* activity of essential oils by paper disc diffusion

Candida spp.	No (%)	Essential Oil[a] (%)	Thyme		Pennyroyal		Lemon	
			25°C	37°C	25°C	37°C	25°C	37°C
C. albicans	34(80.95)	50	52 ±2.3 [b]	56±1.5	32±2.2	44±1.1	11.5±0.6	13±0.3
		25	34±0.1	39±0.8	21.5±0.3	28±0.3	10.2±0.6	10±1.1
		12.5	22.2±0.5	24±1	15.3±0.6	24±1	<6.4	<6.4
		6.25	19±0.4	16.6±0.6	7.6±0.2	21±1.1	<6.4	<6.4
		3.12	13.1±0.6	14±0.4	<6.4	10±0.2	- [c]	-
		1.56	10.9±0.2	11.4±0.5	<6.4	13±0.7	-	-
		0.78	6.8±0.3	7.8±0.3	-	-	-	-
		0.39	<6.4	<6.4	-	-	-	-
C. glabrata	1(2.3)	50	56±0.1	53.8±0.3	28±0.4	32±0.3	10±0.2	10±0.3
		25	33±0.2	41.5±0.1	20±0.3	26±1.2	8.1±0.2	9±0.1
		12.5	24±0.3	27.4±1	12.7±0.1	18±0.2	<6.4	<6.4
		6.25	21.3±0.1	18.3±0.3	10.4±2	9±0.1	<6.4	<6.4
		3.12	14.7±0.2	12.6±0.8	7.2±0.4	<6.4	-	-
		1.56	9.4±0.1	7.8±0.3	<6.4	<6.4	-	-
		0.78	7.2±0.2	<6.4	-	-	-	-
		0.39	<6.4	<6.4	-	-	-	-
C. krusei	6(14.23)	50	47±0.1	51±1.3	36±1.2	50±1.3	13±0.4	11±0.4
		25	36±0.9	40.3±0.3	27±0.7	46±1.8	11.2±0.4	9±0.1
		12.5	27.4±0.1	23±0.3	14±0.3	42±0.5	9.3±0.1	<6.4
		6.25	21.3±0.4	16±0.4	11.3±0	12±0.2	<6.4	<6.4
		3.12	14.2±0.3	12.6±0.3	7.1±0.3	<6.4	-	-
		1.56	10.9±0.1	6.9±0.4	<6.4	<6.4	-	-
		0.78	6.6±0.3	<6.4	-	-	-	-
		0.39	<6.4	<6.4	-	-	-	-
C. albicans (PTCC5027)	1(2.3)	50	53±0.4	49.8±0.3	35±0.2	35±0.3	12.7±0.1	14±0.3
		25	39.8±0.3	39±0.3	23±0.7	22±0.2	8.4±0	11±0.1
		12.5	28±0.2	32±0.9	17±0.2	16±0.3	<6.4	8±0.4
		6.25	18±0.2	26.7±0.1	10.1±0.6	12±0.8	<6.4	<6.4
		3.12	13.4±0.2	18.4±1	8.2±0.2	8±0.2	-	-
		1.56	11.1±0.3	10.2±1	<6.4	<6.4	-	-
		0.78	7.2±0.4	7.3±0.1	-	-	-	-
		0.39	<6.4	<6.4	-	-	-	-

a. Essential oils were emulsified in hexane, b. Inhibition zone, mm in diameter ± standard deviation, c. According to the primary experiment, we did not use these dilutions for pennyroyal and lemon essential oils



A B C D

Fig. 1: Anti-*Candida* activity of essential oils and antifungal drugs at 25°C. A, thyme essential oil; B, amphotericin B; C, fluconazole; D, pennyroyal essential oil

Table 2: Sensitivity of *Candida* species to essential oils

Zone (mm)	Essential oil	Concentration (%) 50	25	12.5	6.25	3.12	1.56	0.78	0.39
<7[a]	Thyme[a]	0[d]	0	0	0	0	2	4	8
	Pennyroyal[b]	0	0	0	0	3	7	-	-
	Lemon[c]	0	0	6	8	-	-	-	-
7-11[b]	Thyme	0	0	0	0	0	5	4	0
	Pennyroyal	0	0	0	4	5	0	-	-
	Lemon	2	6	2	0	-	-	-	-
>11[c]	Thyme	8	8	8	8	8	1	0	0
	Pennyroyal	8	8	8	4	0	1	-	-
	Lemon	6	2	0	0	-	-	-	-

a. Resistant, b. Moderate, c. Sensitive, d. Thyme essential oil used in concentration between 0.39-50%, e. Pennyroyal essential oil used in concentration between 1.56-50% f. Lemon essential oil used in concentration between 6.25-50%. g. The numbers of *Candida* species counted in two temperatures and two series

Table 3: The anti-*Candida* activity of antifungal drugs by paper disc diffusion

Drug	Concentration (µg/ml)	*C. albicans*		*C. glabrata*		*C. krusei*		*C. albicans* (PTCC5027)	
		25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C
Amphotericin B	512	16±1.2[a]	18±0.3	17±0.6	17±0.2	16.5±0.4	17±0.3	18±1.2	17±0.3
	256	14±0.2	17±0.3	14.2±0.2	16±0.4	13.8±0.3	16±1.1	14±0.4	17±0.9
	128	13.6±0.2	16±0.8	13.1±0.8	15±0.1	12.9±0.1	15±0.7	10±0.2	15±0.5
	64	11.4±0.9	14±0.1	11.8±1	13±0.3	11±0.2	14±0.3	9.3±0.1	14±0.6
	32	8.5±1	14±0.3	9.9±0.1	12.5±1	9.4±0.1	13±0.1	8.4±0	13±0.3
	16	7.4±0	11±0.2	8±0.4	12±0.3	8.1±0.6	10±0.2	7.8±0.3	12±0.8
	8	6.6±0	8±0.6	6.4±0	9±0.1	7.2±0.4	9±0.1	6.4±0	8±0.3
	4	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4
Fluconazole	2048	44.5±0.7	43±0.3	30±1.1	25.2±1.6	44±1.1	33±0.2	35±0.1	28.7±0.1
	1024	31±1.2	32±1	16.5±0.4	17.5±0.7	30.5±0.7	21±0.4	23.5±0.8	17±0.1
	512	20.5±0.4	28±0.7	11.7±0.1	14±0	18.5±0.5	13.5±0.3	15±0.8	13±11
	256	16.5±0.1	23.5±0.6	7.2±0.5	8.5±0.1	14±1.3	7.7±0.4	12±0.4	8.5±0.4
	128	11±0.3	16.5±1.7	<6.4	7.2±0.1	7.7±0.1	<6.4	9±0.2	7.7±0.4
	64	<6.4	12.5±0.5	<6.4	<6.4	6.8±0.2	<6.4	7.5±0.4	<6.4
	32	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4
	16	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4

a. Inhibition zone, mm in diameter ± standard deviation. Number of isolates were tested: *C. albicans*: 34 (80.95%), *C. krusei*: 6(14.29%), and *C. glabrata* and standard *C. albicans*, each one: 1(2.38%)

Table 4: Sensitivity of *Candida* species[a] to antifungal drugs

Zone mm	Drug	Drug concentration µg/ml					
		512	256	128	64	32	16
<7[b]	Amphotericin B	0	0	0	0	0	0
	Fluconazole	0	0	2	6	8	8
7-11[c]	Amphotericin B	0	0	1	1	4	5
	Fluconazole	0	4	4	1	0	0
>11[d]	Amphotericin B	8	8	7	7	4	3
	Fluconazole	8	4	2	1	0	0

a. Number of isolates were tested: *C. albicans*: 34 (80.95%), *C. krusei*: 6(14.29%), and *C. glabrata* and standard *C. albicans*, each one: 1(2.38%).b. Resistant, c. Moderate, d. Sensitive

The anti-*Candida* activities of essential oils on different *Candida* species were slightly different. These results were reached by studies done on the *Candida* strain isolated from the infants and the standard strain. *Candida albicans* was the strongest species, because it is the most common agent in candidiasis and different treatments given patients made it more resistant [11,13]. Essential oils had a better inhibitory effect at 37°C than 25°C ($P<0.001$). The inhibitory effect of the essential oils increased when the concentration of essential oil was changed. These results have been reported in other studies [8,14,15].

Amphotericin B had better inhibitory effects at lower concentrations than fluconazole. This result was similar to those of other studies [13,16], They indicated that amphotericin B had more inhibitory effect against to *C. albicans* than fluconazole. Fluconazole had zone inhibition bigger than amphotericin B with the same concentration. This matter may be due to type of solvent. Because the primary solvent for fluconazole was water, so these preparation can induce diffuse of this drug better than amphotericin B [2,16].

Although use of micro-dilution in determining anti-*Candida* activity is the best way [14,16], other methods such as agar diffusion and paper disc diffusion could be utilized as a screening method or in combination with a micro-dilution assay [8,9,17,18]. In this study the anti-*Candida* activities of essential oils were assessed by measuring the diameter of the zone around the paper discs (6.4 mm in diameter); in other studies discs with different diameters were used [11,12,17,19]. The smaller or bigger zone around the colony is related to the sensitivity or resistance of the fungi to the tested materials. Because there are differences in measurement of the transparent zone and also because different amounts and dilutions of the tested materials were used, comparisons are difficult [9,11,18].

There are limitations in the use of antifungal drugs, such as resistance [20], toxic effects [21] and allergic reactions [10]. Further, in some countries plants are used in traditional medicine [6,19,22]. For these reasons we need to compare antifungal drugs with plant-derived ones, which are cheaper, safer and more nature-friendly [9,10,19]. Although the anti-*Candida* activities of essential oils are different due to factors such as environmental conditions, extraction methods and non-standardized processing, we will hope for the futures of these products [4,5]. Essential oils could be used as herbal medicine for some diseases, after, of course, further experiments with more strains of *Candida* species in animal models and human volunteers.

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