

## **Original article**

# **Laboratory study of anticandidal activity of thyme, pennyroyal and lemon essential oils by micro dilution method**

**Saeid Mahdavi Omran\*<sup>1</sup>, Seddigeh Esmaeilzadeh<sup>2</sup>, Zahra Rahmani<sup>3</sup>**

<sup>1</sup>*Department of Medical Parasitology and Mycology, School of Medicine, Babol University of Medical Sciences, Babol, Iran*

<sup>2</sup>*Department of Obstetrics and Gynecology, Yahyanejad Hospital, School of Medicine, Babol University of Medical Sciences, Babol, Iran*

<sup>3</sup>*School of Medicine, Mazandarn University of Medical Sciences, Sari, Iran*

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### **Abstract**

**Introduction and objective:** Patients with candidiasis could be treated with antifungal drugs. Due to side effects and drug resistance, many studies have been conducted to investigate essential oils' antifungal effects. The aim of the present study was to determine anticandidal activity of some essential oils.

**Materials and methods:** In this study, the effect of thyme, pennyroyal and lemon essential oils on *Candida* isolated from vulvovaginal infection were investigated experimentally by using micro dilution method. Serial dilution of thyme (0.002-4%), pennyroyal (0.004-8%) and lemon (0.15-32%) essential oils accompanying to controls and also amphotericin B (0.008-16µg/ml) and fluconazole (1-2048µg/ml) were used.

**Results:** Thyme's essential oils had the most anti-candidal activity followed by pennyroyal. *Candida albicans* was the most sensitive species (0.008-0.062%). Thyme and lemon essential oils had the most (0.008-0.271%) and least (1-32%) anticandidal activities, respectively. The candidal growth was inhibited by amphotericin B at 1µg/ml; whereas the anticandidal activity of fluconazole was 29.647-978.824µg/ml. the effect of thyme essential oil was similar to amphotericin B.

**Conclusion:** Because of the strong anti-candidal effects of thyme which was proved through *in vitro* tests in our study, it is proposed to do further research in the treatment of candidiasis with thyme experimentally.

**Keywords:** Anticandidal, Thyme, Pennyroyal, Lemon, Essential oils, Micro dilution

### **\*Address for correspondence:**

Saeid Mahdavi Omran, Department of Medical Parasitology and Mycology, School of Medicine, Babol University of Medical Sciences, Babol, Iran, Postal Code: 47176-47745; Tel: +98111 2199591-5;

Fax: +98111 2199936; Email: mahdavi@s@yahoo.co.uk

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## Introduction

Candidiasis is a common opportunistic fungal disease in the entire world, and vulvovaginal candidiasis is also one of the most frequent infections in female genital tract with a high incidence [1-3]. Although the majority of candidiasis are caused by *Candida albicans*, non-*albicans* species of *Candida* such as *C. glabrata* and *C. krusei* which are less susceptible to azoles derivatives have been reported with increasing frequency [4]. Amphotericin B and fluconazole, two important agents against human pathogenic fungi, have side effects as well as toxic effects. Thus there is the need for better, novel antifungal agents against infections by some fungi, especially *Candida* species [5,6].

In the last two decades, some research has focused on using herbal components, which have fewer side effects [7,8]. Meanwhile, extracting effective drug components from these herbs, such as herbal essential oils, which are used as antimicrobial, antiviral, and antifungal agents, is increasing [9,10]. Plant-derived essential oils are natural, cheaper, and safer, thus; plant extracts are preferred in the cure of fungal infections [11]. Some of these plants' essential oils are used as a remedy for headaches, arthritis, and also to treat skin discoloration, infectious, and parasitic diseases [12].

Antifungal *in vitro* susceptibility testing should provide useful information for selecting the most active drug against etiological agents [13]. Several oils of plant origin have been used in ancient medicine against some infections in the world and also in Iran, many years ago [14-16]. These compound play essential roles in traditional medicine especially in developing countries [5,9]. The aim of this study is to investigate the anti-candidal activity of some herbal

essential oils on *Candida* isolated from vulvovaginal candidiasis. They will then be compared with anti-fungal drugs to find out their efficacy in the prevention and treatment of the diseases.

## Materials and methods

### Fungi

In this study, we used three species of *Candida*, including *C. albicans*, *C. krusei*, and *C. glabrata* isolated from candidal vaginitis and one species of standard *C. albicans* (PTCC 5027) (purchased from Scientific and Industrial Investigations Organization of Iran). The yeast cells were harvested from Sabouraud dextrose agar, supplemented with chloramphenicol (SC) (Merck Co., Germany), and then counted and adjusted to a final concentration ( $5 \times 10^2$ - $2.5 \times 10^3$  cells/ml). The ratio of live yeast to counted yeast was obtained by a certain amount of culture of counted suspension in petri dishes containing the SC medium.

### Essential oils

The essential oils of thyme (bath number 84.3), pennyroyal (bath number 84.2) and lemon (bath number 84.1) herbs were purchased from Barij Essans Pharmaceutical Co. (Kashan, Iran). These essential oils were kept in a dark flask at 4°C.

### Drugs

Amphotericin B (Sigma Co., USA) and fluconazole (Fuji Co. Japan) were dissolved in Dimethyl sulfoxide (DMSO, Sigma Co. USA) and distilled water, respectively. 128 mg/ml dilution of these drugs divided into little volumes and kept in -70°C before use [17].

### Micro dilution method

Procedures for both micro dilution testing of yeasts against drugs and different concentration of essential oils were performed following the steps established by the National Committee for Clinical Laboratory Standards [17]. Medium RPMI1640 (with L-glutamine, without bicarbonate, and with a pH indicator, Gibco Co., United States) with NaHCO<sub>3</sub>, 2gr/l, (Merck Co., Germany) and 3-(*N*-morpholino, propane sulfonic acid) buffer (34.53g/l, Sigma Co. USA) were used as the culture medium. According to primary experience, we had chosen two times dilution in 12 concentrations of essential oils, including lemon (0.15-32%), pennyroyal (0.004-8%) and thyme (0.002-4%). We also used 0.008-16 µg/ml of amphotericin B and 1-2048µg/ml of fluconazole, to comparison with the essential oil.

On the other hand positive control (containing fungus and medium without essential oils or drugs), negative controls (medium without fungus or drug), and solvent control for amphotericin B (with the highest concentration of DMSO and fungus) were used. All these steps were duplicated. Plates were kept at 37°C in an incubator (Termo Shaker PST-60, Boeco, Germany) and 25°C in another incubator (Heidolph Unimax 1010 Inkubator, Germany) at 150rpm for 48 hours.

After the end of incubation, 10µl of transparent component of wells were added to 6cm in diameter plates containing the SC medium. To spread the suspension very well, one milliliter of melted agaros (45 to 50°C) was used. Plates were kept in an incubator at 37°C for 48 hours and minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined according to the colony count of each plate and they were

compared to initial concentration of fungus. It has been assumed as MFC, if fungal growth inhibited by essential oils; 90% and 50% inhibition of colony growth were MIC<sub>90</sub> and MIC<sub>50</sub>, respectively.

#### *Statistical analysis*

Statistical analysis system (SAS) in general linear model (GLM) was used to compare means and Duncan's multiple range test was evaluated in 1% Level.

#### **Results**

Anti-candidal activity of essential oils with different concentrations (which was done in a pilot study), revealed that candidal growth is influenced by different concentrations of essential oil, the type of essential oil and temperature. Among them, thyme had the most effect and the least anti-candidal activity was observed in lemon essential oil (Table 1 and Fig. 1). Result of variance analysis showed that thyme with the least concentration has the highest inhibitory activity. The most sensitive species was *C. albicans*, followed by *C. krusei* and *C. glabrata* species. There was no significant difference between anti-candidal activities of essential oils. Amphotericin B was more effective in lower concentrations than fluconazole (Table 2).

The analysis showed that there was a statistical significant difference between anti-candidal activity of drugs ( $P<0.0001$ ), but there were no significant statistical differences between *Candida* species and temperatures. Anti-candidal activities of essential oils and drugs on candidal growth (after adjustment of their scales) were analyzed based on the statistical model mentioned above. According to this method there were statistical differences between drugs and essential oils effects and inhibition of candidal growth ( $P<0.0001$ ).

**Table 1:** Anti-candidal activity of some essential oils against to *Candida* species

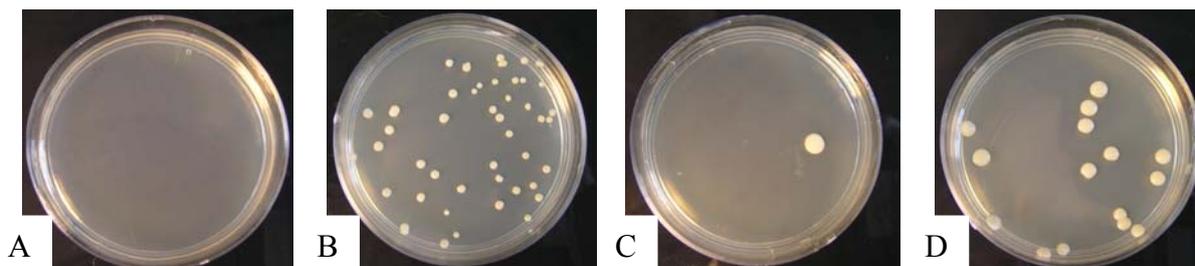
<i>Candida</i> species	No	Essential oil	MIC <sub>50</sub>		MIC <sub>90</sub>		MFC	
			25°C	37°C	25°C	37°C	25°C	37°C
<i>C. albicans</i>	34	Thyme	0.004 <sup>a</sup> ±0.002	0.002±0.000	0.008±0.003	0.002±0.000	0.062±0.025	0.004±0.002
		Pennyroyal	0.246±0.109	0.016±0.005	0.441±0.185	0.064±0.029	2.059±0.814	0.551±0.228
		Lemon	1.941±0.649	1±0.301	8±2.412	7.765±2.594	15.765±5.015	15.765±6.066
<i>C. glabrata</i>	1	Thyme	0.002	0.03	0.002	0.06	0.008	0.124
		Pennyroyal	0.008	0.25	0.03	0.25	0.25	0.5
		Lemon	8	4	16	8	32	16
<i>C. krusei</i>	6	Thyme	0.069±0.046	0.018±0.01	0.146±0.086	0.016±0.011	0.271±0.122	0.03±0.017
		Pennyroyal	0.27±0.0122	0.067±0.031	0.229±0.146	0.145±0.085	0.5±0.273	0.5±0.387
		Lemon	1.917±1.201	3.667±2.338	8±4.382	7.67±4.803	14.667±9.352	15.333±9.606
<i>C. albicans</i> (PTCC 5027)	1	Thyme	0.008	0.004	0.03	0.008	0.06	0.015
		Pennyroyal	0.015	0.004	0.03	0.008	0.125	0.03
		Lemon	4	4	8	4	16	8

<sup>a</sup>The scale of all of data are according to volume/volume (%); and mean± standard deviation, except for *Candida glabrata* and standard *C. albicans*.

**Table 2:** Anti-candidal activity of antifungal drugs against to *Candida* species

<i>Candida</i> species	No	Drugs	MIC <sub>50</sub>		MIC <sub>90</sub>		MFC	
			25°C	37°C	25°C	37°C	25°C	37°C
<i>C. albicans</i>	34	Amphotericin B	0.25 <sup>a</sup> ±0.09	0.471±0.17	0.485±0.12	0.463±0.18	2.059±0.81	0.971±0.39
		Fluconazole	31.06±12.42	29.65±13.14	252.24±80.25	233.41±119.87	978.82±147.41	978.82±147.41
<i>C. glabrata</i>	1	Amphotericin B	0.25	0.5	0.5	1	2	4
		Fluconazole	32	16	128	256	1024	1024
<i>C. krusei</i>	6	Amphotericin B	0.25±0.14	0.5±0.27	0.5±0.27	0.917±0.58	0.916±0.21	1.833±1.17
		Fluconazole	7.332±1.63	28.67±2.34	554.66±251.7	106.67±33.05	938.66±209.02	938.66±29.04
<i>C. albicans</i> (PTCC 5027)	1	Amphotericin B	0.06	0.125	0.125	0.5	1	1
		Fluconazole	8	32	64	256	1024	1024

<sup>a</sup>The scale of all of data are according to µg/ml; and mean± standard deviation, except for *Candida glabrata* and standard *C. albicans*.



**Fig. 1:** Anti-candidal activity of thyme essential oil (0.002%v/v) at 37°C against *Candida albicans*; A, negative control; B, positive control; C, MIC<sub>90</sub> and D, MIC<sub>50</sub>

### Discussion

The results of this study, which aimed to determine anti-candidal activity of herbal essential oils of thyme, pennyroyal, and lemon on growth of *Candida* species isolated from candidal vaginitis, showed that the most anti-candidal activity belongs to thyme. These results agree with previous study reports that noted the inhibition effect of thyme essential oil was 2mg/ml [18]. Results of another work which was performed by using paper disc diffusion, was in agreement with the present study; they showed that thyme essential oil had the most inhibitory effect on *Candida* spp. [19].

Some research which studied anti-candidal activity of thymol (effectiveness material of thyme), showed these results, too [20]. It has been seen that thyme essential oil is better than other oil derivatives from plant [5]. Lemon essential oil had low anticandidal activity in our study. In a study, *C. albicans* was inhibited by lemon oil at 0.5-2%, these differences may be due to the source of *Candida*, because we isolated these *Candida* spp. from vulvovaginitis. Other reasons such as temperature and humidity can influence the compound of essential oils, and the anticandidal activity [21].

In another study, differences between anti-candidal activity of thyme and lemon were found [22]. Lemon essential oil MIC was more effective in other studies [5,22].

This may be caused by using whole lemon fruit as an essential oil (acid components will increase antifungal activity) [5]. In the present study, we used *Candida* species isolated from vaginitis, which may have an increased resistance to drugs due to incomplete treatments. Some genetic differences between standard and wild species may be the causes of these differences [5,23].

The result of this study is different from the report that suggested MIC of 23 herbal essential oils affects three varieties of *C. albicans*, which were isolated from infants and adults [8]. The result of MIC and MFC of amphotericin B and fluconazole agreed with reports on research performed on *C. albicans* [5,17,24]. Almost all of yeasts were resistant against fluconazole in this study; it is different from some studies, due to the use of drug as self mediated [24]. Some of these plants' essential oils had different anticandidal activities, which may be due to differences in species and variety of *Candida* [7]. Although there are some differences on inhibitory rates among *Candida* species *in vitro* [24,25], in this study, like other studies, there was no significant species differences [5].

Regardless of the type of the essential oil, there are significant differences between the interactions of temperature and anti-candidal activity of drugs on candidal growth. This means that for one species

25°C was more effective and for the other one 37°C was more effective on inhibition of candidal growth. Results of the present study showed that amphotericin B in minimum concentration had an inhibitory effect, and thyme essential oil was the next effective order. Although fluconazole had less inhibitory effect on candidal growth, there were no statistical significant differences between thyme essential oil and fluconazole.

These results conform to the reports from previous researchers who studied about this subject. This shows the difference of inhibitory potential of these components on different fungi [18,22,24]. The results show a favorable inhibitory effect of thyme essential oil on candidal growth. According to this result, anticandidal components of thyme in different forms be used to prevent candidal infection experimentally. Meanwhile, the effect of these components on other fungi should be investigated.

### Conclusion

We concluded that some essential oils were used in this study had anticandidal activities, related to *Candida* species.

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