



## ***Mentha piperita* extract, a potential antifungal agent against *Candida albicans* and *Candida krusei***

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### **Abstract**

Along with the increasing number of drug resistance of *Candida* sp. and also high mortality of candidiasis, a study on alternative treatments must be carried out. *Mentha piperita* is known to have potent antifungal activity. Therefore, this study was conducted to explore the antifungal effect and its mechanism of action of *Mentha piperita* extract (peppermint oil) on the growth of *Candida albicans* ATCC 10231 and ATCC 90028, and *Candida krusei*. Peppermint oil was extracted from *Mentha piperita* leaf by steam distillation. Disk diffusion method was conducted according to CLSI M44 and germ tube test was done to assess its antifungal activity and mechanism. Inhibition zone of peppermint oil against *Candida albicans* ATCC 10231 was 31.67 mm, *Candida albicans* ATCC 90028 was 17 mm and *Candida krusei* was 43.67 mm. Germ tube test showed a reduction of germ tube formation with the increase of peppermint oil concentration ( $p=0.024$ ). In conclusion, *Mentha piperita* extract has the potential to be developed as an antifungal against *Candida albicans* and *Candida krusei*. Its mechanism of action could be inhibition of germ tube formation.

**Key words** – candidiasis – drug resistance – germ tube – inhibition zone – peppermint oil

### **Introduction**

*Candida* sp. is a commensal fungus on the skin, mouth, and gastrointestinal tract that can change over into pathogen when the balance of normal flora inside the body or a person's immune defense decline (Berkow & Lockhart 2017). The spectrum of infection by *Candida* sp. is very wide, starting from a non-fatal infection and mucocutaneous infection to the invasive ones and infiltrates the internal organs (Nelwan 2014). Systemic candidiasis may occur when *Candida* sp. enters the bloodstream and the body's immune response is inadequate to withstand the growth of *Candida* sp. (Mitchell 2016). Lots of systemic candidiasis occur in the intensive care unit (ICU) on patients with neutropenia or the patient that uses intravascular catheter infected by the microflora from the patient's skin or from the skin of the nurse who handles the patient.

Epidemiological data from several countries stated that the incidence of systemic candidiasis in ICU reached 33-35% and the mortality rate was around 30-62% (Bouza & Muñoz 2008). In Barcelona, most cases (51%) of systemic candidiasis caused by *Candida albicans*, 23% by *Candida*

*parapsilosis*, 10% by *Candida tropicalis*, and the rest by *Candida glabrata* or *Candida krusei*. The mortality rate was higher in patients with systemic candidiasis caused by non-*albicans* species. While in Taiwan, *C. glabrata* is the second most common species causing systemic candidiasis, and 11% of *C. glabrata* are resistant to fluconazole (Bouza & Muñoz 2008).

Treatment of candidiasis is done with the administration of antifungal drugs. The development of antifungal drugs has begun since 1950, but nowadays, some species of *Candida* have undergone resistance to antifungal drugs, mainly from the derivative of azole (Roemer & Krysan 2014, Candrasari 2014). *Candida albicans* has the lowest incidence of azole resistance which is about 0-5%, while *C. krusei* has intrinsic resistance to fluconazole with a percentage of 96.6% and *C. glabrata* has an incident to the resistance of azole by 7.8% (Whaley et al. 2017). These facts prove that the development of antifungal drugs is not as rhythmic with clinical needs (Roemer & Krysan 2014). Antifungal resistance is one cause of treatment failure for fungal infection and it is also a challenge for health workers (Candrasari 2014). Therefore, it is necessary to find new antifungal drug.

Herbal plants are one example of alternative medicine that can be used by the community and have benefits in treating several kinds of diseases (Rasool Hassan 2012). One of the herbs that can be extracted into essential oil is mint leaf (*Mentha piperita*) (Saharkhiz et al. 2012). Essential oils of mint or peppermint oil can provide an antifungal effect on *C. albicans* (Saharkhiz et al. 2012, Loolaie et al. 2017). Menthol is a compound of peppermint oil which allegedly acted as an antifungal (Balakrishnan 2015). Thus, aim of this study was to observe antifungal activity of peppermint oil against *C. albicans* and *C. krusei*, especially in *C. krusei*, which has intrinsic resistance to fluconazole. This study also aimed to determine the mechanism of action of peppermint oil against *C. albicans*.

## Materials & Methods

This experimental study was conducted in the laboratory facilities of Department of Biochemistry and Parasitology, School of Medicine and Health Sciences, Universitas Katolik Indonesia Atma Jaya from June 2019 to November 2019. Ethical clearance was received from the ethical committee with the number of 12/06/KEP-FKUAJ/2019.

### Extraction method and bioactive compounds test

The sample used was mint leaf (*Mentha piperita*) from Hijos Farm, Indonesia. The extraction method was done by steam distillation. Tests of the bioactive compounds of menthol and eugenol were conducted on the obtained peppermint oil. Test for menthol was conducted using vanillin-sulfuric acid reagent, while the test for eugenol using potassium hydroxide reagent. In the menthol test, vanillin-sulfuric acid reagent would change mixture color into yellow. The positive result was marked by the change in color into purple after a few drops of water. In the eugenol test, a positive result was obtained when a bright white to a yellow color mixture with flocculated mass was formed.

### Fungal strain

We used *C. albicans* (ATCC 10231), *C. albicans* ATCC 90028 and *C. krusei* wild-type in this study. Susceptibility profile of *C. krusei* was determined using the Sensititre™ YeastOne™ (Thermo Scientific, United States) microdilution kit and confirmed that this strain was resistant to fluconazole. All three fungi were obtained from the collection of Department of Parasitology, School of Medicine and Health Sciences, Universitas Katolik Indonesia Atma Jaya.

### Disk diffusion test

Disk diffusion test was conducted in accordance with the Clinical and Laboratory Standards Institute (CLSI) M44 with slight modification (CLSI 2018). In brief, sabouraud dextrose agar (SDA, Oxoid, United Kingdom) was used as the medium instead of mueller hinton agar. Disk diffusion test was performed for three repetitions (triplo) and the mean was calculated to determine

the inhibition zone. Peppermint oil was impregnated on a blank disk (Oxoid, United Kingdom) for one hour at 37°C. Fluconazole 25µg and ketoconazole 15µg disks (Liofilchem, Italy) were used as the positive control, while blank disk was used as the negative control.

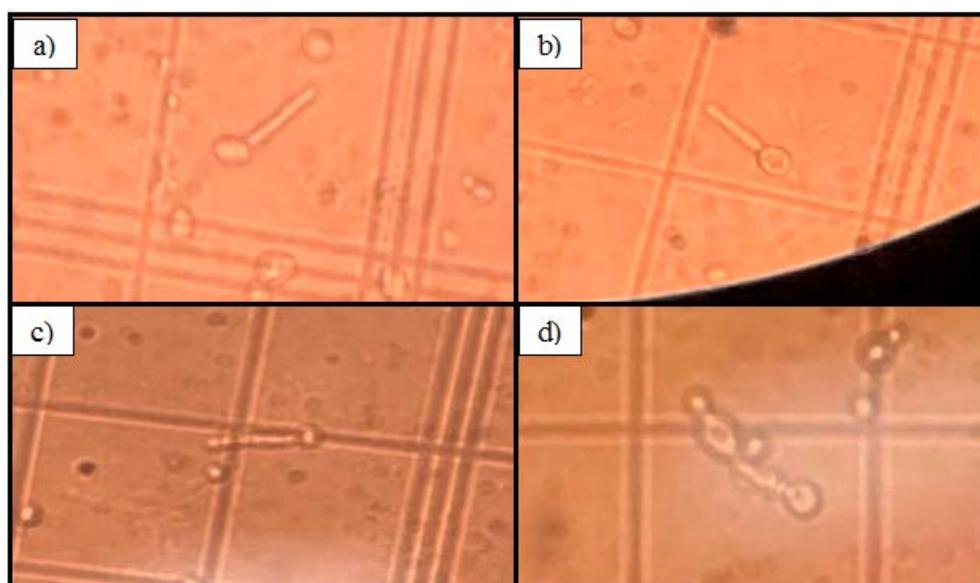
### Germ tube method to test the mechanism of peppermint oil against *C. albicans*

The white part of chicken eggs and sabouraud dextrose broth (SDB, Oxoid, England) were used as a medium for germ tube test. Egg whites, SDB, 1 µL of dimethyl sulfoxide (DMSO, Emsure, United States) and peppermint oil (0%, 25%, 50% and 75%) were mixed in a test tube and incubated for 15 min at 37°C (Table 1). The suspension of *C. albicans* ATCC 10231 (McFarland 3.5) was inserted into each test tube and then homogenized using a vortex. The mixture was then incubated for three hours at 37°C. Three repetitions were done for each concentration.

**Table 1** Mixture for germ tube test

Peppermint oil concentration	<i>C. albicans</i> Suspension (in µL)	White Egg (in µL)	DMSO (in µL)	Peppermint oil (in µL)	SDB (in µL)	Total volume (in µL)
0%	100	133	0	0	767	1000
25%	100	133	1	250	516	1000
50%	100	133	1	500	266	1000
75%	100	133	1	750	18	1000

After incubation for three hours, 10 µl of the mixture was dropped in the improved neubauer counting chamber and the amount of germ tube formed was counted on 25 small squares (central square) as many as three times repetitions for each tube. Germ tube characteristic was tail-like structure arise form yeast cell. Its structure was similar to pseudohyphae without proximal constriction (Aryal et al. 2015). Examples of pseudohyphae and germ tube are shown in Fig. 1.



**Fig. 1** – Difference of germ tube and pseudohyphae. a, b, c are germ tube or hyphae (tail-like structure with no constriction), while d is a pseudohyphae (with proximal constriction).

### Data analysis

Data processing was done with the Statistical Product and Service Solution (SPSS) for windows version 25.0. Data were analyzed using one-way ANOVA statistical test or Kruskal-Wallis test, depended on data normality. One-way ANOVA test or Kruskal-Wallis was significant when  $p \leq 0.05$ .

## Results

### Bioactive compound test

Ten ml of peppermint oil was obtained from 750g of mint leaves. The obtained peppermint oil had a transparent liquid green-yellowish color. The bioactive compound test with vanillin-sulfuric acid reagent showed color changes to purple. It signified that the tested peppermint oil contained menthol. While the test of bioactive compounds with potassium hydroxide showed discoloration into a cloudy-white color but there was no flocculated mass. This indicated that peppermint oil tested did not contain eugenol.

### Disk diffusion test

Inhibition zones obtained were shown in Table 2. Mean inhibition zone of peppermint oil against *C. albicans* ATCC 90028 was 16 mm, *C. albicans* ATCC 10231 was 31.67 mm, and *C. krusei* was 43.67 mm. Inhibition zone of fluconazole against both *C. albicans* ATCC 90028 and ATCC 10231 was around 28 mm and 23 mm, while for ketoconazole against *C. krusei* was 45.33 mm.

**Table 2** Results of disk diffusion test against *C. albicans* and *C. krusei* (in mm)

	<i>C. albicans</i> ATCC 90028				<i>C. albicans</i> ATCC 10231				<i>C. krusei</i> wild-type			
	I	II	III	Mean	I	II	III	Mean	I	II	III	Mean
Peppermint oil	16	20	15	16	30	33	32	31.67	45	46	50	43.67
Fluconazole	27	28	29	28	22	24	23	23	-	-	-	-
Ketoconazole	-	-	-	-	-	-	-	-	46	45	45	45.33
Aquadest	0	0	0	0	0	0	0	0	0	0	0	0

### Germ tube test

Germ tube formed in 0% concentration of peppermint oil had an average of 10.17, and it decreased to an average of 8.67, 5.17, and 2.00 for 25%, 50%, and 75% concentration, respectively with  $p=0.024$  (Table 3). Based on the Post-Hoc Kruskal-Wallis test result, the difference of peppermint oil concentration of 0% and 75% had  $p=0.030$ . There was no significant difference between other concentration groups.

**Table 3** Germ tube test

Peppermint oil concentration	Number of germ tube formed									Mean germ tube formed	p
	Tube I			Tube II			Tube III				
	A	B	C	A	B	C	A	B	C		
0%	9	4	6	5	1	10	6	6	5	10.17	0.024
25%	2	3	5	2	2	5	2	3	3	8.67	
50%	0	1	1	0	0	1	0	2	0	5.17	
75%	0	0	0	0	0	0	0	0	0	2.00	

## Discussion

We successfully extracted peppermint oil from the mint leaves by steam distillation. Bioactive compound tests were conducted to ensure that *Mentha* species used was *Mentha piperita*. *Mentha piperita* and *Mentha spicata* are the two most common species that are easily found. Both species are distinguished from the menthol composition. *Mentha piperita* contains 30-70% menthol, while *Mentha spicata* contains <1% menthol (Cirlini et al. 2016, Fatih et al. 2017). A positive result on the test of menthol compound confirmed that our mint leaf was *Mentha piperita* (Loolaie et al. 2017, Saharkhiz et al. 2012, Rajkowska et al. 2017). The bioactive compound test of eugenol was done to ensure that peppermint oil obtained through the extraction process was a pure

peppermint oil unmixed by other compounds. Eugenol should not be found in peppermint oil, but it is found in plants or essential oil of cloves, pepper, betel leaves, cinnamon, saffron, nutmeg, and thyme (Khalil et al. 2017, Giuliani 2014). The presence of menthol content may indicate that *Mentha piperita* has antimicrobial, antifungal, and antiviral activities (Loolaie et al. 2017).

Disk diffusion test was conducted on *C. albicans* ATCC 90028, *C. albicans* ATCC 10231, and *C. krusei*. The results of disk diffusion of peppermint oil against *C. albicans* and *C. krusei* showed relatively large inhibition zone. It indicates that peppermint oil has the potential to inhibit the growth of both fungal species and strains. Based on Devkotte et al. (2005), the average inhibition zone obtained on the results of peppermint oil against *C. albicans* ATCC 10231 was 18.73 mm. Research by Agarwal et al. (2010) got an average of 22.2 mm inhibition zone on the disk diffusion test for peppermint oil against *C. albicans* (strain was not mentioned). In this study, an average of 17 mm inhibition zone for *C. albicans* ATCC 90028 and 31.67 for *C. albicans* ATCC 10231 were observed for peppermint oil. The results of disk diffusion obtained in this study had a slight difference with previous studies because this method is influenced by the volatility of substances, disk size, the number of substances that incubated on the disk, the disk ability to absorb a substance, the type of disk to be used, the pH and volume in order, and fungal strains used (Scorzoni et al. 2007). Based on research by Nascimento et al. (2007), the essential oil activity test as an antifungal can provide less consistent results due to several factors such as the volatility, water solubility, and viscosity of a substance.

In this study, it was found that there was a reduction of germ tube formation started at a concentration of 25%. According to the Post-Hoc Kruskal-Wallis test results, germ tube number was decline significantly at a concentration of 75% compared to 0%. Based on research by Pinto et al. (2013), the effect of essential oils on the inhibition of germ tube formation might occur due to oxidative stress which affected the enzymatic activity and affected the mitochondrial potential membrane of a cell, which could result in inhibition of the growth of a cell and even cell death. Saharkhiz et al. (2012) stated that peppermint oil could penetrate the cell membrane of *Candida*. Antifungal effect of peppermint oil can be explained through the cytotoxic effect that causes damage and changes in membrane fluidity (Rajkowska et al. 2017).

In this study, assessment of mechanism of peppermint oil against *C. albicans* test results has some limitations. This study does not calculate the number of yeast cells present in each concentration. The number of yeast cells was appeared to decrease and peppermint oil may have a fungicidal effect on *C. albicans*. Menthol was not purely isolated and the amount of menthol was not calculated, so we could not ensure that menthol was the reason for antifungal activity.

In conclusion, peppermint oil could inhibit the growth of *Candida albicans* and *Candida krusei*. Inhibition of the germ tube formation is suspected to be one of the mechanisms of peppermint oil in inhibiting the growth of *Candida albicans*.

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