In vitro antifungal activity of ethanolic and ethyl acetate extract of mint leaves (Mentha piperita L.) against Candida albicans

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Abstract. The oral cavity health of human can describe their health condition. The oral candidiasis disease caused by Candida albicans, an opportunistic mycoflora in mouth cavity, can be treated with several antifungal drugs including nystatin, ketoconazole, and fluconazole. Mint (Mentha piperita L) leaves are one of the herbal ingredients used as a refresher in natural mouthwash and known to have antibacterial activity. This study aimed to determine the antifungal activity of ethanolic and ethyl acetate extract of mint leaves against C. albicans. The mint leaves were macerated with ethanol and ethyl acetate solvents, macerate obtained were then evaporated on a rotary evaporator. Ethanolic and ethyl acetate extract of mint leaves were tested for antifungal activity against C. albicans using the Kirby - Bauer method. The concentration of extract used was 40%, 60% and 80% w/v and 25,000 μg ketoconazole used as the positive control, while 100% dimethyl sulfoxide used as the negative control. The results showed that antifungal activity of ethanolic and ethyl acetate extract of mint leaves against C. albicans was weaker than 25,000 µg of ketoconazole. The strongest antifungal activity of ethyl acetate extract found at 80% w/v concentration with 9.95 mm diameter of inhibition zone, while the inhibition zone diameter formed in 80% w/v, ethanolic extract was 7.65 mm.

1. Introduction
The oral cavity health of human can describe their health condition — the oral candidiasis disease caused by Candida albicans. This disease described the weakness of the systemic defense mechanism, changes in normal flora or poor oral hygiene [1]. In recent years, many antifungal drugs have been found for the treatment of diseases caused by fungal infections. Oral candidiasis has been treated using synthetic antifungal drugs, including nystatin, ketoconazole, and fluconazole. However, improper use of antifungal drugs can cause side effects and microbial resistance to these antifungal [2].

Currently, back to nature movement has become a new world trend by consuming medicines from herbal ingredients as an alternative medical treatment, due to the lack of side effects [3]. One of the herbal ingredients that are often used is mint as an ingredient in producing toothpaste, mouthwash and other.

This study aimed to determine the antifungal activity of ethanolic extract and ethyl acetate extract of mint leaves against C. albicans. This research was expected to provide information about the potential of mint leaves as an herbal plant that has antifungal activity against C. albicans.

2. Materials and Methods
2.1. Production of simplisia and extraction
Mint leaves of 3-4 weeks old were obtained from Getasan Village, Kopeng District, Central Java. 4,000 grams of fresh mint leaves were sorted, obtained 3,100 grams of clean mint leaves. Mint leaves were washed, cut and oven dried at 60°C. Simplisia was mashed using a blender [4]. Simplisia were soaked in ethanol and ethyl acetate (1:5) for maceration, shaking at 120 rpm for 48 hours, allowed to stand for 24 hours and filtered. Macerate obtained were evaporated using a rotary evaporator, while the pulp was re-macerated for two times [5].

2.2. Phytochemical analysis
The test solution was prepared by dissolved 0.4 gram of ethanolic and ethyl acetate extract into 4 ml of distilled water containing tween 80. Alkaloid test was carried out by adding 1 mL of test solution with 1 mL of 2% HCl, heated for 5 minutes and then filtered. The filtrate obtained was dripped with 2-3 drops of Dragendorff reagent. The presence of alkaloid compounds was indicated by the red or orange precipitate [6]. The saponin test was carried out by mixing 1 mL of test solution with 1 mL of distilled water, shaken for 10 seconds. The presence of saponin compounds was indicated by the appearance of foam as high as 1 cm for not less than 10 minutes and still presence when dripped with concentrated HCl [7]. The flavonoid test was carried out with 1 mL test solution which was added with 2 mL of methanol, 0.1 gram of Mg powder and 5 drops of concentrated HCl. The flavonoid compounds were indicated by the appearance of red or orange color [6]. The tannin test was carried out with 1 mL of test solution which was added a few drops of FeCl₃. Tannin compounds were shown by the change of color to dark blue or greenish black [8].

2.3. Yeast culture and purity examination of C. albicans
C. albicans culture was obtained from the Microbiology Laboratory of Dr. Kariadi Hospital Semarang, maintained on Yeast Malt Agar (YMA) slant agar, kept at 28 °C. The culture purity examined under a microscope with 1000x magnification.

2.4. Antifungal activity test of ethanolic and ethyl acetate extract of mint leaves
The antifungal activity test was carried out using Kirby-Buer disk diffusion method on Muller Hinton Agar with C. albicans suspension (6.3 x 10⁷ CFU / ml) as test microbes. The Whatman Grade AA disc paper with a diameter of 6 mm was dripped with 20 μL of 40%, 60%, and 80% w/v mint leaves extracts of, then put on the agar plate surface which already inoculated with the test microbes. The plates were then incubated at 37°C for 24 hours. The antifungal activity examined by measuring the diameter of the inhibition zone around the disc paper [10].

2.5. Statistic analysis
The antifungal activity of ethanolic and ethyl acetate extract of mint leaves was analyzed using One-Way ANOVA and Post Hoc Duncan using SPSS 15.0.

3. Result and Discussions
The mint leaves powder had a brownish green color (Figure 1.). 200 grams powder were obtained from 3,100 grams of fresh mint leaves, with a simplisia yield of 6.45% w/v. The powder was macerated with ethanol and ethyl acetate solvents, resulting in a blackish green macerate (Figure 2). The macerate was evaporated using a rotary evaporator and produced a blackish green paste (Figure 3.). The yield of ethanolic and ethyl acetate extract of mint leaves were 6.82% and 3.37% w/v.
The ethanolic extract and ethyl acetate extract mint leaves have antifungal activity against *C. albicans* based on its chemical compound, indicated by the formation of inhibition zone (Figure 5. and Figure 6.). The ethanolic and ethyl acetate extract of mint leaves both contained alkaloid and saponin compounds (Table 1.). According to Sujana *et al.* (2013) [5] and Bansode and Chavan (2014) [11], the ethanolic and ethyl acetate extract of mint leaves containing alkaloid and saponin compounds. The alkaloid compound is known to have antimicrobial activity by inhibiting DNA and RNA polymerase, and cell respiration [12]. Saponin was a compound that acts as an antifungal by reducing the surface tension of the sterol membrane which causes leakage of cell membranes with intracellular fluid discharge so that fungal cells die [13].

**Table 1. Phytochemical test results of ethanolic and ethyl acetate extract of mint leaves**

| Phytochemical Test | Reagent          | Result   | Notation          |
|--------------------|------------------|----------|-------------------|
|                    |                  |          | Ethanol Extract   |
|                    |                  |          | Ethyl Acetate     |

Figure 1. Mint leaves powder

Figure 2. Macerates of mint leaves extract (a) ethanolic extract (b) ethyl acetate extract

Figure 3. Mint leaves extract (a) ethanolic extract (b) ethyl acetate extract
Alkaloids

HCl 2% Dragendorff

Red sediment is formed + +

Saponin

Concentrated HCl Aquadest

A stable foam is formed as high as 1 cm + +

Flavonoid

Methanol Mg Powder Concentrated HCl

The color does not turn into red or orange - -

Tannin

FeCl₃

The color does not change to dark blue or greenish black - -

| Compound | Extract | Description |
|----------|---------|-------------|
| Alkaloids | HCl 2% Dragendorff | Red sediment is formed |
| Saponin | Concentrated HCl Aquadest | A stable foam is formed as high as 1 cm |
| Flavonoid | Methanol Mg Powder Concentrated HCl | The color does not turn into red or orange |
| Tannin | FeCl₃ | The color does not change to dark blue or greenish black |

Information:
+: contain compound
- : does not contain compound

Figure 4. Documentation of antifungal activity of mint leaves against *C. albicans* (a) ethanolic extract (b) ethyl acetate extract

Ketoconazole 25,000 µg formed an inhibitory zone in the test, indicating that the drug was able to inhibit *C. albicans* (Figure 6.). Ketoconazole is a broad spectrum antifungal with a mechanism to inhibit the synthesis of ergosterol which is a component of the fungal cell membrane [14]. Ethanolic extract and ethyl acetate extract of mint leaf formed a smaller and clearer inhibition zone compared to 25,000 µg ketoconazole. The strongest antifungal activity test results were 80% concentration of ethyl acetate extract. Ethanolic extract of mint leaves at a concentration of 40%; 60%; 80% inhibition zones were formed sequentially 6.45 mm (47% compared to positive controls); 6.9 mm (51% compared to positive controls); 7.65 mm (56% compared to positive controls). Ethyl acetate extract of mint leaves at a concentration of 40%; 60%; 80% inhibition zones were formed sequentially by 7.48 mm (55% compared to positive controls); 8.58 mm (63% compared to positive controls); 9.95 (73% compared to positive controls). The concentration of mint leaves extract was increase make the inhibition zone, and the antifungal activity of the extract was increase too.
Figure 5. Antifungal activity of ethanolic and ethyl acetate extract of mint leaves towards *C. albicans* (K+: Ketoconazole 25,000 µg and K-: DMSO 100%). Numbers with the same letter in the same extract indicate the insignificant result. The data then analyzed using One-Way ANOVA and Post Hoc Duncan, showed that ethanolic extract of mint leaves with the concentration of 40%, 60%, and 80% had no significant effect on antifungal activity. Ethyl acetate extract of mint leaves with a concentration of 80% had no significance effect with concentrations of 40% and 60% so that 80% concentration had an insignificant effect on antifungal activity.

Antimicrobial inhibitory power is categorized based on inhibition zone diameter including weak (6-10 mm), medium (11-20 mm) and strong (21-30 mm) [15]. Based on the category of antimicrobial inhibitory power, it can be concluded that ethanolic extract and ethyl acetate extract of mint leaves are included antifungal with weak inhibitory power category.

4. Conclusion
Ethanolic extract and ethyl acetate extract of mint leaves have antifungal activity towards *C. albicans*, with a weak inhibition category. The extract has lower antifungal activity compared to the positive control of 25,000 µg/mL ketoconazole.

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