In vitro Antifungal Activity of Methanolic and Chloroform Mint Leaves (*Mentha piperita* L.) Extracts Against *Candida albicans*

K Y Wenji¹, I Rukmi¹ and A Suprihadi¹

¹Department of Biology, Faculty of Science and Mathematics, Diponegoro University
Jl. Prof. H. Soedarto, S.H. Tembalang, Semarang 50275, Indonesia
E-mail: wenjikristiyunitaa@gmail.com

Abstract. Oral health is an important thing that must be prioritized because all intake is first processed in the mouth. The most common disease found in the oral cavity is candidiasis, caused by *Candida albicans* which is an opportunistic intraoral pathogen that inhabits the oral cavity. *Peppermint* (*Mentha piperita* L.) is one of the herbal plants which has proven in laboratory scale have antifungal activity. This research aimed to know the antifungal activity of methanolic and chloroform extract of *M. Piperita* L. leaves against *C. albicans*. Mint leaf extract was obtained by maceration method using methanol and chloroform as solvents. The extract concentration for antifungal activity test were 40%; 60% and 80% w/v solution in DMSO 100%. The Kirby-Bauer method was used to examine the antifungal activity of both extracts. The results showed that both methanolic and chloroform extract has antifungal activity against *C. albicans*. The antifungal of methanolic better than the chloroform extract. Both extracts at all concentration showed a greater antifungal activity compared to 25,000 µg ketoconazole as a positive control. The best antifungal activity of methanolic mint leaves extracts found at 80% concentration. It is no different in antifungal activity of chloroform mint leaves extract at all concentration tested.

1. Introduction

Oral health is an important thing that must be prioritized because all intake is first processed in the mouth before being digested in other digestive organs. One of the diseases in the oral cavity is oral candidiasis caused by *Candida albicans* which is the most common opportunistic intraoral pathogen inhabiting the oral cavity [1], [2].

Controlling the population of *C. albicans* is one way to prevent infection in the oral cavity caused by yeast. The main types of antifungals used for the treatment of *Candida* infections are azoles, polyenes, and echinocandins [3]. However, the use of antifungals still has weaknesses, for example, less effective due to resistance and the presence of adverse side effects.

Traditional medicine is believed to cure various diseases without giving side effects to those who consume them, and the go back nature movement also supports the use of herbs. *Mentha piperita* L. is included in the Labiatae family that grows particularly well in environments with high water-holding capacity soil [4]. Mint is widely used as a popular ingredient for chewing gum, toothpaste, tea and is also used to treat a sick stomach or to improve digestion [5]. *M. piperita* L. extract on a laboratory scale kills several types of bacteria, fungi, and viruses so that the contents can be developed as antibacterial, antifungal and antiviral [6], [7].
Recent years mint leaves are commonly used as mouthwashes with a mixture of bay leaves because both are believed to kill bacteria that inhabit the mouth and safe for health. Karlina's research [8] states that natural mouthwashes made from bay and mint leaves can be used to cleanse the mouth. Based on that, this research will examine the antifungal activity of mint leaves extract specifically without being combined with other plants against the yeast C. albicans which is a normal flora in the oral cavity.

2. Material and method

2.1. Plant material and extraction

Mint plants four weeks old were obtained from Getasan Village, Kopeng District, Semarang Regency, Indonesia. The mint leaves were sorted, then washed with running water to remove impurities. The leaves were cut into pieces and oven drying at 60°C. The simplistic obtained were mashed using a warring blender to reduce particle size [9].

The Simplisia of mint leaves were then macerated using methanol and chloroform solvents (1:5 w/v). The content of erlens was mixed at room temperature by a shaker with 120 rpm for 48 hours and was then filtered. The pulps were then remacerated two times. The solvents obtained was evaporated using a rotary evaporator at 40-60°C [10], [11].

2.2. Phytochemical analyses

The alkaloids, flavonoids, tannins, and saponins components of the extract were examined. The test solution used were 1 gram of mint leaf extract in 10 mL aquadest in addition of 500 μL tween 80.

2.2.1. Alkaloid test

To 1 mL of test solution, 1 mL of 2% HCl was added, then heated for 5 minutes and filtered. The filtrate obtained was dripped with 2-3 drops of Dragendorff reagent. The presence of alkaloid compounds is indicated by the formation of reddish brown precipitate [12].

2.2.2. Flavonoid test

To 1 mL of test solution, 2 mL of methanol and 0.1 g of Mg powder was added followed by five drops addition of concentrated hydrochloric acid. The presence of flavonoids is indicated by the formation of red or orange colour [12].

2.2.3. Tannin test

To 1 mL of test solution, iron (III) chloride reagent was dripped a few. The formation of dark blue or blackish greencolour indicates the presence of tannin compounds [13].

2.2.4. Saponin test

1 mL of test solution was added to aquadest and then shaken vertically for 10 seconds. Presence of saponin compounds are shown by the formation of foam as high as 1cm for approximately 10 minutes and when dripped by HCl, the foam is still form [14].

2.3. Microbial cultures and growth conditions

C. albicans culture was obtained from Microbiology Laboratory of RSUD Kariyadi, Semarang. C. albicans was maintained on Yeast Malt Agar (YMA) at 28°C for 24 hours, then stored in a refrigerator.

2.3. Microscopic observation of C. albicans

Microscopic observations of C. albicans done by simple staining using methylene blue as a coloring agent to find out that the purity of culture. The colony of C. albicans was taken slightly using ose and then flattened on the glass preparation which had been dropped by methylene blue.

2.4. Antifungal activity assay by disc diffusion method

C. albicans suspension with a density of 6.3 x 10⁷ CFU/mL was grown on Mueller Hinton Agar (MHA) using a cotton swab method. Whatman Grade AA sterile disc paper of 6 mm diameter were dripped with 20 μL mint leaves extract with a concentration 40%, 60% and 80% w/v, then placed on the surface of
MHA which already inoculated with *C. albicans*. The plates were incubated at 28° C for 24 hours. 25.000 ug ketoconazole was used as a positive control to determine the sensitivity of the fungal strains respectively and 100% dimethylsulfoxide was used as a negative control. Antifungal activity was evaluated by measuring the diameter of the inhibition zone around the disc [15].

2.5. Statistical Analysis
Data of antifungal activity were analyzed using SPSS 16.0 with 95% confidence level.

3. Result and discussion
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/w. The powder were macerated with methanol and chloroform solvents, resulting a blackish green color paste and has a distinctive smell. The percentage rendemen of mint leaves crude methanolic extract of was 4,68% w/w, while chloroform extract was 5,08% w/w. Selection of solvents based on extraction requirements, this study used methanol and chloroform solvents. Methanol is a universal solvent and chloroform is a semipolar solvent [16], [17].

![Figure 1. Mint leaves powder.](image)

![Figure 2. Mint leave extracts: a. Methanolic extract; b. Chloroform extract.](image)

**Table 1.** The phytochemical analysis in the crude methanolic and chloroform mint leaf extract.

| Phyto-constituents | Extracts        | Phytochemical | Observation                              | Inference |
|-------------------|-----------------|---------------|-----------------------------------------|-----------|
| Alkaloids         | Methanol, Chloroform | Dragendorff   | Reddish brown precipitate               | +         |
| Flavonoids        | Methanol, Chloroform | Mg powder, HCl | No change in colour of the extract      | -         |
| Tanins            | Methanol, Chloroform | FeCl₃        | No change in colour of the extract      | -         |
| Saponins          | Methanol, Chloroform | HCl          | Formation of persistent froth           | +         |
The results of the phytochemical analysis of crude methanolic and chloroform mint leaf extract showed the presence of alkaloids and saponins compounds (Table 1). The phytochemical test result in this study have different result with other literature, but according to Park et al. [18] the content of phytochemical compounds in mint plants is different for each species. Several factors such as physiological variations, environmental conditions, geographical differences and genetic factors cause differences in the composition of chemical compounds in mint plants. The mechanism of alkaloids as antifungi includes inducing apoptosis in fungi and inhibiting Candida Drug Resistance gene (CDR1), while mechanism of saponins as antifungi is through membrane destruction and induction of apoptosis in fungi. The destruction of cell membranes through the binding of ergosterol will result in loss of function of the cell membrane [19]. This proves that the inhibition zones of C. albicans formed may be due to the presence of alkaloids and saponins that contained in both extracts methanol and chloroform extract of mint leaves.

Figure 3. Morphology of C. albicans at 1000x magnification using methylene blue.

C. albicans observed were isolates grown on YMA. Microscopic observations showed that C. albicans yeast cells were oval and consisted of mother cells, daughter cells, and shoots (Figure 3). The evaluation of antifungal activity by disc diffusion method indicated that C. albicans strain tested showed growth inhibition toward both plant extract. Negative control of 100% DMSO does not show an inhibitory zone (Figure 4) because DMSO does not inhibit microbial growth [20]. This indicates that the inhibitory zone formed by mint leaf extract comes from the phytochemical constituents. The positive control has a smaller inhibitory zone diameter than mint leaves extracts, it shows that methanol and chloroform extract of mint leaves have more effective antifungal activity (Figure 4). This is presumably because ketoconazole only has one mechanism to inhibit C. albicans by inhibiting demethylation of lanosterol to ergosterol [21] while extracts of methanol and chloroform of mint leaves have more than one inhibitory mechanism from phytochemical constituents.
Figure 4. Antifungal activity of mint leaves with concentration 40% w/v (upper right), 60% w/v (lower right) and 80% w/v (lower left): a. Chloroform extract; b. Methanol extract.

Higher inhibition zone showed at methanol compared to chloroform extract (Figure 5). This is presumably because the chloroform extract of mint leaves has a thicker structure than the methanol extract of mint leaves, so that the inhibitory zones formed tend to be smaller. According to Madan & Sigh [22] the higher the viscosity of a compound, the more difficult for active substance of the drug compound will comes out. C. albicans showed a higher inhibition zone by increased concentration (Figure 5). This was similar to the results of Maleki et al. [23] that the growth of microbial will mostly decrease with increasing antimicrobial concentration. The higher the extract concentration, the greater the amount of antimicrobial compounds released, thereby facilitating the penetration of these compounds into microbial cells.

Figure 5. Antifungal activity of crude extract methanol and chloroform of mint leaves (Blue bar: methanol extract; Green bar: chloroform extract).

The highest average inhibition zone diameter in methanol and chloroform extracts of mint leaves was 10 mm and 9.28 mm at concentration of 80% w/v, the antifungal activity formed was relatively
weak according to Morales et al. statement [24], antimicrobial activity by active ingredients grouped into 3 categories, weak (6-10 mm), medium (11-20 mm) and strong activity (21-30 mm). Inhibition zone in methanol extract has a wider diameter than the result of Pramila et al. [25], this is suspected because there were differences in the amount of extract that was dripped on the disc paper, the phytochemical content in the extract and the density of Candida suspension used in the test.

Results of ANOVA test showed that the data of methanol and chloroform extracts mint leaves had a significant difference (p < 0.05). Duncan’s test results on the methanol extract of mint leaves showed a different significant between 80% concentration with a concentration of 40% and 60%. While test results on chloroform extract of mint leaves showed no different effect between all concentrations.

4. Conclusion
Methanol and chloroform extracts of mint leaves have antifungal activity against yeast *C. albicans*, with the most effective concentration of 80% in methanol extract.

5. Acknowledge
The author wish to acknowledge gratitude to Mrs. Isworo Rukmi, M.Kes from Biology Departement of Diponegoro University for financial support and providing the research facilities.

Reference
[1] Gaines S, James T C, Folan M, Baird A W, and O’Farrelly C 2003 *J Microbiol Methods* 54 315–23
[2] Blankenship J R and Mitchell A P 2006 *Curr Opin Microbiol* 9 588–94
[3] Silva S, Rodrigues C F, Araujo D, Rodrigues M E and Henriques M 2017 *Journal of Fungi* 3(8)
[4] Loolaei M, Moasefi N, Rasouli H and Adibi H. 2017. *Arch Clin Microbiol* 8(4) 54
[5] Capello G, Spezzaferro M, Grossi L, Manzoli L and Marzio L 2007 *Digestive Liver Dis* 39 530–536
[6] Raja R R 2012 *Research Journal of Medicinal Plant* 6 (3) 203–213
[7] Sigh R, Shushni M A M and Belkheir A 2015 *Arabian Journal of Chemistry* 8 322–328
[8] Karlina L 2016 Efektivitas Kombinasi Ekstrak Daun Salam dan Daun Mint sebagai Obat Kumur Alami *Publikasi Ilmiah* (Surakarta)
[9] BPOM RI 2013 *Petunjuk Operasional Penerapan Cara Pembuatan Obat yang Baik Jilid I.* (Badan Pengawas Obat dan Makanan Republik Indonesia, Jakarta)
[10] Istiqomah 2013 Perbandingan Metode Ekstraksi Maserasi dan Sokletasi terhadap Kadar Piperin Buah Cabe Jawa (*Piperis reticulata* fructus) *Skripsi* (Program Studi Farmasi, UIN Jakarta)
[11] Julian M I 2008 Pengaruh Pemberian Ekstrak Etanol Daun Gandarusa (*Jasminum officinale* L.) terhadap Kadar Asam Urat dalam Darah Tikus Putih yang Dibuat Hiperurisemia dalam Kalium Oksonat *Skripsi* (Departemen Farmasi, UI Depok)
[12] Harborne J B 1996 Metode Fitokimia Alih bahasa: Padmawinata K dan Soediro I (Institut Teknologi Bandung, Bandung)
[13] Robinson T 1991 *Kandungan Organik Tumbuhan Tinggi* (Institut Teknologi Bandung press, Bandung)
[14] Depkes RI 1995 Farmakope Indonesia (Departemen Kesehatan Republik Indonesia, Jakarta)
[15] Pramila D M, Xavier R, Marimuthu K, Kathiresan S, Khoo M L, Senthilkumar M, Sathy K and Sreeramanan S 2012 *Journal of Medicinal Plants Research* 6 (2) 331–335
[16] Astarina N W G, Astuiti K W, Warditiani M K 2013 Skrinining Fitokimia Ekstrak Metanol Rimpang Bangle (*Zingiber purpuratum* Roxb.) *Jurnal Farmasi Udayana* 10 1–6
[17] Rais I R 2014 *Pharmaciana* 4 (1) 85–92
[18] Park Y J, Baskar T B, Yeo S K, Arasu M V and Al-Dhabi N A 2016 Composition of volatile compounds and in vitro antimicrobial activity of nine *mentha* spp *Springer plus* 5 1628
[19] Lee H and Lee D G 2015 Mode of Action of Bioactive Phytochemical, Plant Secondary Metabolites, Possessing Antimicrobial Properties *The Battle Against Microbial Pathogens:*
Basic Science, Technological Advances and Educational Programs (A. Méndez-Vilas, Ed.), Formatex 185–192

[20] Hatijah S, Husain D R, Sartini 2013 Bioaktivitas Minyak Atsiri Umbi Lapis Bawang Merah Allium cepa L. Lokal Asal Bima Terhadap Bakteri Streptococcus mutans Penyebab Karies Gigi Skripsi (Universitas Hasanuddin, Makasar)

[21] Mycek M J, Harvey R A, Champe P C 2001 Farmakologi Ulasan Bergambar edisi 2. Alih bahasa: Agus A, Widya Medika (Jakarta)

[22] Madan J and Singh R 2010 International Journal of Pharmaceutical Sciences 2 551–515

[23] Maleki D, Seyyeednejad S M, Damabi M N and Motamedi H 2008 Journal of Biological Science 11 (9) 1286–1289

[24] Morales G, Sierra P, Mancilla A, Paredes A, Loyola L A, Gallardo O and Bourquez J 2003 J. Chile Chem 48 (2) 35–41

[25] Pramila D M, Xavier R, Marimuthu K, Kathiresan S, Khoo M L, Senthilkumar M., Sathy K and Sreeramanan S 2012 Journal of Medicinal Plants Research 6 (2) 331–335