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Membrane cell disruption of *Candida albicans* by Masoyi bark essential oil

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ABSTRACT

Essential oils distilled from the bark of *Massoia aromatica* Becc. (Masoyi) has been known from our previous research as a potential anti-candidiasis towards both planktonic and biofilm states. This study aims to determine Masoyi essential oil's antifungal mechanism of action towards *Candida albicans* ATCC 10231. Observation of the antifungal mechanism towards *C. albicans* was performed by *Scanning Electron Microscope* (SEM) and *Transmission Electron Microscope* (TEM) following the Masoyi essential oil application in sub-minimum inhibition concentration, in comparison to untreated cells as the control. Considering that both planktonic and biofilm of *C. albicans* were influenced, the effect on farnesol production was also observed using thin layer chromatography of the microbial supernatant. Following SEM and TEM results, the effect on membrane permeability seems to play a role in the antifungal activity. To support the result, an experiment using influx Propidium Iodide Test was performed. TLC densitometry and GC-MS analysis showed that C-10 Massoialactone is a major constituent of Masoyi oil. Observation of *C. albicans* cells by using SEM showed cell shrinkage, which may be due to disruption in membrane cell permeability. TEM confirmed the membrane cell disruption, which causes cytoplasmic leakage. The result of the propidium iodide influx test confirmed the damage to membrane permeability. The decrease in farnesol production was observed, which may play a role in the antibiofilm activity of the essential oil.

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INTRODUCTION

Candida albicans is a polymorphic fungus living as a part of human commensal microflora, mostly found in the mouth cavity and digestive tract (Mayer *et al.*, 2013). However, in the last three decades, the case of *C. albicans* infection has increased rapidly in immune-compromised patients such as those who have HIV/AIDS (Samaranayake *et al.*, 2002). Medical treatment with available systemic antifungi gave a positive response, yet the side effects such as fever, vomiting, muscle spasm, and hypotension have limited the therapy. Moreover, Al-Attas and Amro (2010) reported that diabetes mellitus patients having candidiasis are treated with azoles such as fluconazole, ketoconazole, econazole and miconazole. These issues raise the need for new, more effective antifungi with few side effects.

Medicinal plants offer an alternative source of a new chemical entity which may serve as an anti-

infective. One of the plants commonly used in traditional medicine is *Massoia aromatica*Becc (Lauraceae) known by its local name, Masoyi (Rali *et al.*, 2007). The essential oil distilled from the bark has been reported as an effective anti-microbial by inhibiting the growth of the planktonic cell and biofilm of microbial cells (Hamzah *et al.*, 2018, 2020; Pratiwi *et al.*, 2015) and has an immunomodulatory potential of which the C-10 massoialactone plays a role as the active compound (Hertiani *et al.*, 2016; Permanasari *et al.*, 2017). The Masoyi bark essential oil contains lactone compounds of C10 and C12 while C14 (5.6-dihydro-6-nonyl-2H-piran-2-on) was reported in the hardwood (Rali *et al.*, 2007). Our research has revealed that Masoyi oil provides an inhibitory effect on biofilm formation and degradation of multispecies biofilms (unpublished result). In this recent result, we explore the effect of the Masoyi oil on *C. albicans* membrane. We believe that the effect on the cell membrane may interfere with the antifungal activity and further affecting the biofilm formation of the fungi.

MATERIALS AND METHODS

Massoia aromatica barks were collected from Sorong (Papua, Indonesia). Sample identification was performed by Mr. Djoko Santosa (Laboratory of Pharmacognosy, Faculty of Pharmacy UGM). *C. albicans* ATCC 1023 was used for the assay. C-10 massoialactone was isolated from the essential oil, and the structure elucidation was confirmed in the previous study (Permanasari *et al.*, 2017). Other materials used were farnesol standard (Sigma-Aldrich, Germany) and Propidium iodide (Sigma-Aldrich, Germany).

Sample preparation

The dried-pulverized barks were distilled by water-steam distillation method to yield the essential oil.TLC and Gas-Chromatography Mass Spectrometry (GCMS) were performed on the oil to evaluate the content of C-10 massoialactone as the active ingredient as well as to observe the profile of the oil chemical content. GCMS was performed at the Organic chemistry laboratory, Faculty of Mathematical and Life Sciences UGM, while the TLC scanner was provided by the Phytochemistry laboratory, Faculty of Pharmacy, UGM.

Anti candida assays

The oil was first evaluated its inhibitory effect on biofilm growth of *C. albicans* to determine the $MBIC_{50}$ value (minimum sample concentration to reduce biofilm by 50%). Masoyi oil in concentration series of 1%, 0.5%, 0.25%, 0.125% v/v were tested according to Pierce *et al.* (2008). The treatments were divided into three groups, i.e. cells without treatment, cells with the treatment, and another group as a control using Nystatin. The $MBIC_{50}$ value of oil was used to evaluate the effect of the oil on cell membranes using Influx Propidium Iodide (PI) (Lee and Lee, 2014). Transmission electron microscopy (TEM) was performed according to Sangetha *et al.* (2009) while *Scanning electron microscopy* (SEM) was conducted by the modification of methods described by Hess *et al.* (2012); Hamzah *et al.* (2019); Pelzer *et al.* (2012).

The effect on farnesol production was performed according to the method described by Tashiro *et al.* (2012). The test of farnesol production following sample application was observed by using TLC scanner showed the result of TLC with the observation under a lamp of UV 254 nm, in which the concentration of farnesol standard was prepared as follows, 0.5 μL, 1 μL, 2 μL, and 4 μL.

RESULTS AND DISCUSSION

The oil yield obtained from the distillation process was 0.76% v/w. Thin-layer chromatography analysis was done to obtain the chemical profile of masoyi oil compounds. The visualization using UV254 lamp and visible lamp after the application of anisaldehyde H_2SO_4 spray reagent showed three spots with hRf of 37, 50, 70. hRf 37 was identified as C-10 massoialactone (Figure 1). The result of GC-MS analysis (Figure 2). Showed two peaks, of which 95.68% is identified as C-10 massoialactone (SI 93%) and 4.32% as C-8 massoialactone (SI 90%) (NIST2.LIB and WILLEY229).

Note: Stationary phase: silica gel F254; mobile phase: toluene- ethyl acetate (93:7 v/v), elution path 8 cm. Visualization method: (A) annisaldehyde – H_2SO_4, observed under visible light; (B) anisaldehyde – H_2SO_4, observed under UV 366 nm (C) UV 254 nm, before spraying

The result from *C. albicans* middle phase (24 h) biofilm formation inhibition test showed a decrease in the formation of *C. albicans* biofilm following the increase of the Masoyi oil concentration (Figure 3). Nystatin was used as a control showed biofilm inhibition in the middle phase of *C. albicans* by 71.90% $\pm$ 0.96.

The effect of Masoyi oil on*C. albicans* membrane permeability was observed by monitoring the Propidium iodide influx (Figure 4). Propidium Iodide is a fluorescent probe of DNA staining which can only enter the cell membrane if it is damaged or leaked. When the cell membrane was disrupted by

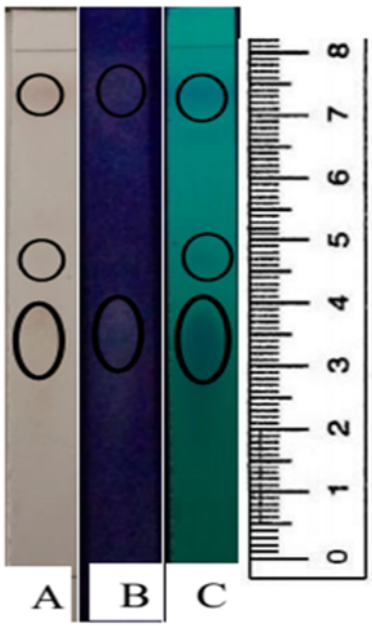

Figure 1: TLC chromatogram of the Masoyi essential oil

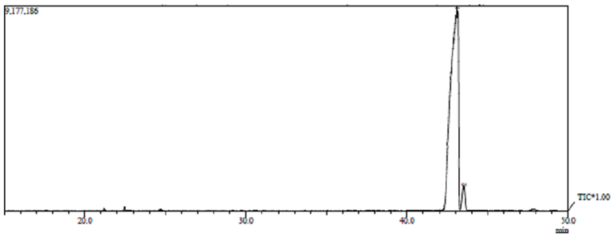

Figure 2: GC profile of the Masoyi essential oil

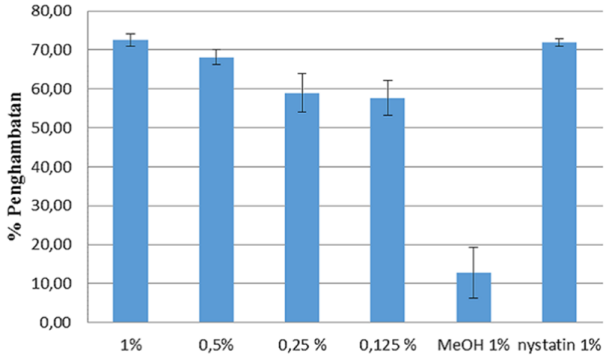

Figure 3: Masoyi oil effect on middle phase biofilm of C. *albicans*

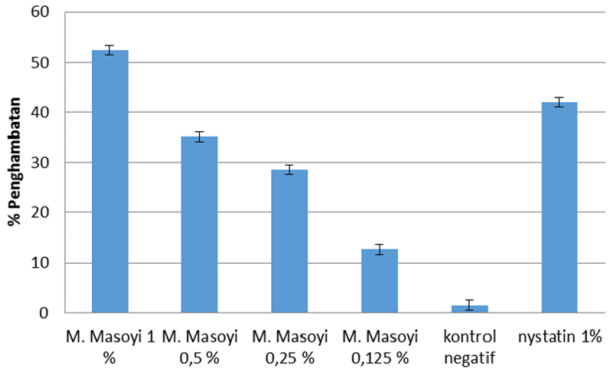

Figure 4: Results of the Propidium Influx assay

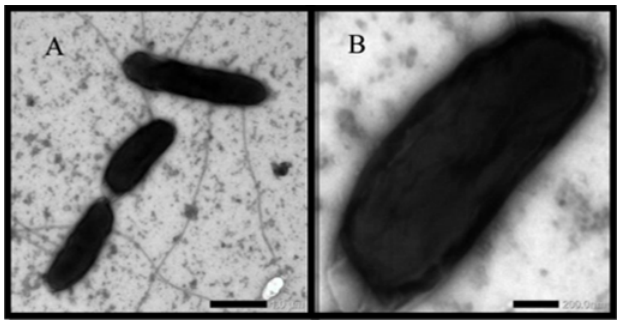

Figure 5: Observation by TEM of untreated cells

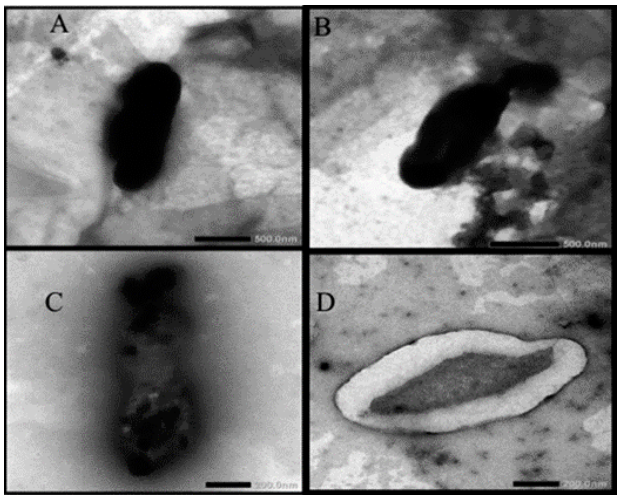

Figure 6: Observation by TEM of cells treated with 0.25% of Masoyi oil

the Masoyi oil, Propidium iodide could seep into the cytoplasm and bindsinto DNA. Through the inclusion of Propidium iodide, the Masoyi oil influenced the cells of *C. albicans* by disrupting the cell membrane and then increase the permeability of the membrane.

The result of TEM analysis showed that *C. albicans* experienced dramatic cell shape changes following the Masoyi oil application. The Masoyi oil increases the membrane cell permeability and causes the cytosol to leak out. Furthermore, the cell membrane of *C. albicans* began to rupture (Figure 5 and Figure 6).

Kishimoto *et al.* (2005) have proposed the anti-candida effect of massoialactones is due to its effect on its respiratory system. Other related compounds showed a nonspecific, anti-candida effect in which their binding to sulfhydryl moiety and enzyme denaturation has led to membrane leakage (Pauli, 2006). Lactones have been reported to bind to sulfhydryl moiety (Pratiwi *et al.*, 2015).

The results from this research were in accordance

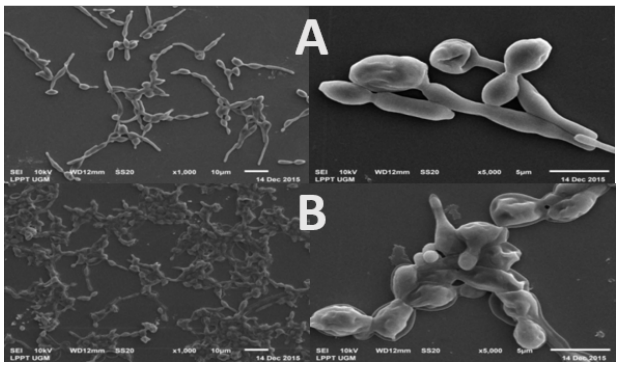

Figure 7: Scanning Electron Microscope micro graph of C. *albicans* Note: A: untreated cells and B:treated cells with Masoyi oil 0.25%

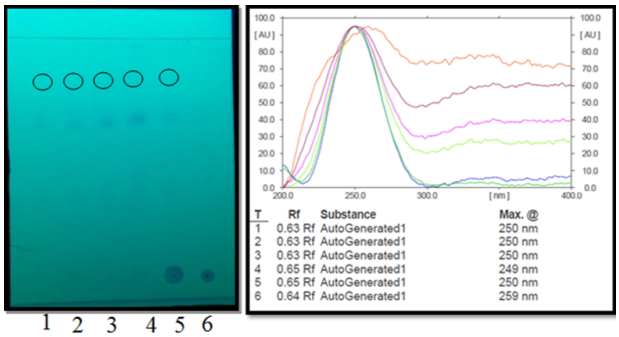

Figure 8: Results of farnesol production taken using theTLC-densitometry method with observations at UV 254 (1with a level of 0.5 μg, 2 with a level of 1 ug, 3 with a level of 2 ug, 4 with a level of 4 ug, 5 cells of C. *albicans* without treatment, 6 cells C. *albicans* with masoyi oil treatment).

with the above-proposed mechanism, which confirmed the effect of the Masoyi oil as a cause of membrane leakage in *C. albicans* .

Scanning Electron Microscope (SEM) and *Transmission Electron Microscope* (TEM) were conducted on the resulted culture following sample application to observe the morphological and structural changes. TEM analyses on *C. albicans* biofilm showed that the Masoyi oil caused less organized biofilm (Figure 6). Ultra structure visualization of untreated *C. albicans* biofilm showed a smooth cell wall. Furthermore, pseudo-hyphae and hyphae were formed (Figure 7A). Biofilm formation inhibition of middle phase culture of *C. albicans* following sample application of 0.25% concentration was indicated by the reduced amount of cell attachment. At the same time, cytosol leakage yet maintains cell wall intact. Cell shrinkage was observed and no true hyphae formed (Figure 7B).

The result of the farnesol production analysis showed the untreated cell produce spot in the TLC, which was similar to that of farnesol standard. The similarity was based on the hRf value and the UV-spectra profile (Figure 8). However, in the cell culture treated with Masoyi oil, no farnesol production was detected. This indicated that the Masoyi oil affects the farnesol production of the *Candida albicans* , which may contribute to its anti-biofilm activity. As we know that farnesol has been reported as the quorum sensing molecule of *C. albicans* to initiate morphological changes from yeast to mycelium, which essential for biofilm formation (Hall *et al.*, 2011).

CONCLUSION

The Masoyi bark essential oil containing the C-10 massoialactone as the major compound showed membrane disruption activity in *C. albicans* led to cell death. Farnesol as the quorum sensing compound in *C. albicans* was observed to be inhibited its production following sample application which may be due to insufficient cell population. This effect was also confirmed by no true hyphae or pseudohyphae formed following sample application.

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