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ANTI FUNGAL ACTIVITY OF METHANOLIC EXTRACT OF *Usnea sp.* AGAINST *Malassezia furfur*

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ABSTRACT

The antifungal activity of methanolic extract of *Usnea sp*. (MEU) against *Malassezia furfur* ATCC 14521, in terms of *in vitro* susceptibility, minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC) were investigated using broth microdilution method with endpoint after 48 h. Time-kill curves were determined at concentrations 0×, 1/2×, 1×, 2×, 4× and 8×MIC. MEU was susceptible against *M. furfur* with diameter clear zone of 34 mm for 1 mg/ml. MIC and MFC values were 16 µg/ml and 64 µg/ml, respectively. Time-kill curve demonstrated that treatment with 4×MIC (64 µg/ml) and 8×MIC (128 µg/ml) of MEU for 4 h and 1 h, respectively, was able to kill 100 % of *M. furfur*. MEU shows potential as an antifungal agent for inhibiting the growth of *M. furfur* ATCC 14521 *in vitro*. MEU might be a useful alternative for treating dandruff, tinea versicolor, tinea capitis, and seborrhoeic dermatitis due to the growth inhibition of *M. furfur.*

Key words: antifungal, *in vitro Malassezia furfur*, methanolic extract of *Usnea sp.* (MEU)

AKTIVITAS ANTI JAMUR EKSTRAK METANOL *Usnea sp.* TERHADAP *Malassezia furfur*

ABSTRAK

Pengujian *in vitro* antijamur ekstrak methanol *Usnea sp*., yang meliputi suseptibilitas, Konsentrat Hambat Minimum (KHM) dan Konsentrat Fungsi Sidal Minimum (KFS) (menggunakan metoda *microdilution* dengan waktu uji 48 jam terhadap *Malassezia furfur*, telah berhasil dilakukan. Pengujian kurva *time-kill* dilakukan pada konsentrasi 0×, 1/2×, 1×, 2×, 4× and 8× KHM selama 48 jam. Hasil pengujian menunjukkan bahwa ekstrak metanol *Usnea sp.* pada konsetrasi 1 mg/ml, suseptibel terhadap *M. furfur* dengan panjang diameter *clear zone* 34 mm. Nilai KHM dan KFS-nya masing-masing adalah 16 µg/ml dan 64 µg/ml. Kurva *time-kill* menunjukkan bahwa *M. furfur* 100% terbunuh dengan konsentrasi 4×KHM (64 µg/ml) selama 4 jam atau dengan konsentrasi 8×KHM(128 µg/ml) selama 1 jam. Hasil-hasil diatas menunjukkan bahwa ekstrak metanol *Usnea sp*., berhasil menghambat pertumbuhan kapang *M. furfur* ATCC 14521. Dengan demikian, ekstrak metanol *Usnea sp*., mempunyai potensi untuk dikembangkan sebagai obat anti ketombe dan obat penyakit-penyakit infeksi jamur lainnya yang disebabkan oleh *M. furfur*.

Kata kunci: antijamur, *in vitro*, *Malassezia furfur*, ekstrak metanol *Usnea sp*.

INTRODUCTION

M. furfur is the causative agent of pityriasis versicolor, tinea capitis, pityriasis folliculitis. Interest in the organism of *M. furfur* has increased considerably in recent years as this yeast has been implicated as the primary cause of the scalp disease known as seborrhoeic dermatitis or dandruff (Bulmer and Bulmer, 1999). More than 50% of the population in the world suffers from some level of dandruff and seborrheic dermatitis (Warner *et al*., 2001). This yeast can also become opportunistic pathogen because of alterations in skin surface environment and host defense (Rosales *et al*., 2005). Moreover, *Malassezia* species have been reported as significant causes of fungal infections in patients with cancer and AIDS (Gabal, 1998; Gupta *et al*., 2000).

Herb extracts are promising sources for new natural antifungal drugs, even though they have relatively mild effect against human pathogenic fungi compared to commercial synthetic antifungal drugs (Hammer *et al*., 1999; Faleiro *et al*., 2003). Furthermore, a dramatic increase in fungal infections has been observed in the last decade, which may be attributed to the augmentation of the number of immunocompromised and immusuppressed patients, more susceptible to opportunistic systemic and superficial mycoses (Rahalison *et al.,* 1994). Fungal resistance to synthetic antibiotics in clinical use is also rising and it often develops rapidly (Metzger & Hofmann, 1997; Rocha *et al*., 2004). Thus, investigation of antifungal extracted from potential herbs should be done.

Usnea or "lumut janggut" (Indonesian) is a unique species of herb because it is created through a symbiotic relationship between lichens and algae. *Usnea* was historically used to treat indigestion because of its bitter taste and activity as a digestive system stimulant (Guo *et al*., 2008). *Usnea* is used to treat abscesses, colds, cough, cystitis, fungal infections (such as athletes foot or ring-worm), gastrointestinal (stomach and intestine) irritations, influenza, sore throats (including strep throat), respiratory infections (sinusitis, bronchitis**,** pneumonia, etc.), skin ulcers, urinary tract infections, and vaginal infections. *Usnea* species have been used as antimicrobial agents in many countries and were being developed as a modern pharmaceutical just prior to the advent of the penicillin antibiotics (Cocchietto *et al*., 2002). The *Usnea* crude extracts have been used throughout the world to treat various ailments, such as pulmonary tuberculosis, pain, fever, wounds, athlete's foot, and other dermal lesions. They have also been used as expectorants, in antibiotic salves, deodorants, and herbal tinctures (Cocchietto *et al*., 2002; Okuyama *et al*., 1995).

Usnea has antifungal activity against *Candida albicans, Trichoderma viride*, and *Cladosporium cladosporioides* (Balaji and Hariharan, 2007; Nanayakkara *et al*., 2005; Thippeswany *et al*., 2011). A few reports regarding to antifungal activity of *Usnea* sp. extract against *M. furfur*, such as supercritical CO_2-extract from *Usnea barbata* (Weckesser *et al*., 2007) and essential oils from 17 plants (Lee & Lee, 2010), have been published. The antifungal activity of *Usnea* extract against *M. furfur* has to extensively investigate. The purpose of this study is to investigate the antifungal activity of methanolic extract of *Usnea sp.* (MEU) against *M. fufur*.

MATERIALS AND METHODS

Growth Conditions and Inoculum Preparation of *Malassezia Furfur*

M. furfur ATCC 14521 was obtained from the American Type Culture Collection (Rockville, MD, USA) and grown on Sabouraud Dextrose Broth (SDB) or Sabouraud Dextrose Agar (SDA) (Difco) supplemented with 1% (v/v) of pure olive oil (Yakuri Pure Chemicals, Kyoto, Japan), following incubation at 35°C during two to seven days. *M. furfur* was maintained on the same medium described above, at 4°C, with subcultures being carried out on a monthly basis. A single colony of *M. furfur* was inoculated into 10 ml of SDB supplemented with 1% of olive oil (SDBO). The culture was incubated at 35°C for 48 h with 200 rev/min agitation. A quantity of 1 ml of 48 h culture was centrifuged (3000 g at 4°C for 1 min), followed by washing the pellets twice with 1 ml of 50 mM pH 7.0 phosphate buffered saline (PBS). Sterile physiological saline was added to give a McFarland turbidity 0.5 at 530 nm, corresponding to approximately 5×10^6 cfu/ml.

Methanol Extract of *Usnea sp*. and Zinc Pyrithione

Usnea sp. or "lumut janggut" samples were purchased from traditional market at Bandung, Indonesia. The dried *Usnea* sp. (100 g) were ground and extracted twice with 400 ml of 100% (v/v) methanol for 48 h at room temperature. Methanolic extracts of *Usnea* sp. (MEU) were filtered with Whatman filter paper NO.2 (Whatman International Ltd., Middlesex, England) and concentrated with a rotary vacuum evaporator (Heidolph

VV2011, Schwabach, Germany) at 50°C, yielding MEU. MEU was dissolved in 10% DMSO to obtain 1024 µg/ml stock solutions. DMSO at 10% was found not to kill the *M. furfur* in this research. Zinc pyrithione (ZPT) (Dongsan Clean & Green, Korea) was dissolved in sterile distillated water to obtain stock solution (128 µg/ml). All the solutions were prepared freshly prior to experiments.

In Vitro Susceptibility Test

MEU was tested for antifungal susceptibility against *M. furfur* using the standard paper disk diffusion assay method as described in the guidelines of CLSI M27-A2 (CLSI, 2002). A 100 µl of inoculum of *M. furfur* prepared as above was spread on SDA plates with a sterile cotton swab. Sterile filter paper discs, 9 mm diameter (Schleicher and Schuell, Dassel, Germany), were placed on the disks and 50 µl of 1 mg/ml (w/v) methanol extract of samples were loaded on the paper discs. A 1 mg/ml of ZPT (a positive control) and 10% of DMSO (a negative control) were included in the assay. The plates were incubated at 35°C for 24-48 h and observed for any clear zones. The experiments were preformed twice to verify the results.

MIC and MFC Determination

In vitro susceptibility tests were performed to evaluate MIC and MFC using broth microdilution method as described in the guidelines of Clinical and Laboratory Standard Institute M27-A2 (CLSI, 2002). *In vitro* antifungal susceptibility testing of yeasts has been standardized using RPMI-1640 based microdilution methods. However, the standardized RPMI-1640 medium does not support growth of the *Malassezia* yeast cells because it lacks the specific lipids they require (Gupta *et al*., 2000). SDBO and SDAO (SDA supplemented with 1% olive oil) media were used for susceptibility testing in this study (Rukayadi *et al*., 2007). Briefly, MIC was determined with the adjusted inoculum suspension of 5×10^6 cfu/ml (a McFarland standard) by diluting 1:10 with SDHO medium to make final inoculum concentration of 5×10^5 cfu/ml. MIC test was carried out by placing 100 µl of inoculum suspension in the 96-wells microplate. One hundred microliters of MEU solution (1024 µg/ml) or ZPT (128 µg/ml) prepared as described above was added to each well in columns 12 and mixed gentl×y. One hundred microliters of the solution in wells in column 12 were then transferred to the adjacent wells in column 11 followed by gentle mixing. This process was continued across the plate ending with the wells of column 3. The final 100 µl aliquot removed from the wells of column 3 after mixing was discarded and the wells in column 2, which were not exposed to antifungal agent, served as positive controls. Wells in column 1, which only content media, served as negative controls. The plates were covered and incubated for 48 h at 37°C. The experiments were performed two times with two replicate wells for each antifungal. MIC was defined as the corresponding concentrations required inhibiting the growth of the *M. fufur* in each column relative to controls grown in the absence of the antifungal agents (column 1). MFC was determined for each antifungal agent as outlined for MIC by removing the media from each well showing no visible growth and subculturing onto SDAO plates by dropping 10 µl of solution. The plates were incubated at 35°C until growth was seen in the growth control plates. MFC was defined as the corresponding concentrations required to kill 100% of the *M. furfur*.

Time-Kill Curves

Time-kill curve were performed in SDHO and SDH medium. The inoculum was adjusted spectrophotometrically to the density of a 0.5 McFarland turbidity standard at 530 nm as described above. The concentrations of MEU prepared were 0×-, 1/2×-, 1×-, 2×-, 4×-, and 8×MIC. The adjusted inoculum suspension, a McFarland standard, of 5×10^6 cfu/ml was diluted 1:10 in SDHO or SDH medium to make final inoculum concentration of 5×10^5 cfu/ml. Each concentration of MEU was diluted 1:10 in SDHO or SDH medium containing 5×10^5 cfu/ml, as prepared previously. This procedure yielded an initial inoculum 4.5×

0^5 cfu/ml. The final MEU concentrations were 0-, 16-, 32-, 64-, 128-, and 256 µg/ml. Cultures (5 ml final volume) were incubated at 35°C with agitation (200 rpm). At predetermined time points (0 min, 5 min, 15 min, 30 min, 1-, 2-, 4-, 8-, 12-, 24-, and 48 h), a 100-µl aliquot was removed and transferred to Effendorf tubes, centrifuged (3,900×g at 4°C for 1 min) and rinsed twice with 0.9 ml of sterile distilled water to obtain xanthorrhizol or zinc pyrithione-free cells. Pellets were suspended in 100 µl of sterile distillated water and serially diluted. An appropriate volume (100 µl, 40 µl, or 20 µl, depending on the dilution and the concentration of MEU) were spread onto SDA plates and incubated at 35°C for 48 h or more (until the colonies were seen on the plates) to determine the numbers of cfu/ml. When the colony counts were expected to be less than that of 1,000 cfu/ml, samples of 20 µl were taken directly from the test solution and plated without dilution. The lower limit of accurate and reproducible colony counts was 30 (1.5 in log 10) cfu/ml. The experiment was repeated with two replicates per experiment.

RESULTS AND DISCUSSSION

Figure 1 shows that MEU was susceptible against *M. furfur* ATCC 14521 with clear zone of 34 mm for 1 mg/ml of MEU. MIC and MFC of MEU to *M. furfur* compared to that of ZPT are summarized in Table 1. MIC and MFC of MEU against *M. furfur* was 16 µg/ml and 64 µg/ml, respectively. Meanwhile those of ZPT were 0.5 and 4 µg/ml, respectively. The killing activity of MEU against *M. furfur* is shown in Figure 2. The fungicidal endpoints for *M. furfur* were reached after 1 h of incubation at 128 µg/ml or after 4 h at 64 µg/ml.

Zinc pyrithione (ZPT) was used as a positive control in comparison with antifungal activity of MEU. ZPT has been used broadly for treating skin disease caused by *Malassezia* including dandruff. Schmidt and Ruhl-Horster (1996) reported that MICs of ZPT against *M. furfur* was between 0.2 to 8 µg/ml. Our result showed that MIC of ZPT against *M. fufur* was 0.5 µg/ml and (Table 1). Thus, susceptibility of ZPT against *M. furfur* is in good agreement with previous report.

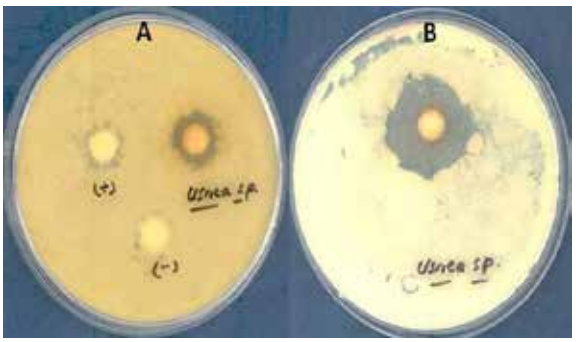

Figure 1. Susceptibility of methanolic extract of *Usnea sp*. (MEU) against *M. furfur* ATCC 14521. (A) Concentration of MEU was 50 µl of 1 mg/ml; (+), 50 µl of 0.1 mg/ml ZPT; and (-) 50 µl of 10% DMSO. (B) 50 µl of 1 mg/ml (diameter clear zone was 3.4 cm or 34 mm).

Table 1. *In vitro* antifungal activity of methanolic extract of *Usnea sp*. (MEU) and zinc pyrithione (ZPT) against *M. furfur* ATCC 145521

Methanolic extract of *Usnea sp*. (MEU) (µg/ml)		Zinc pyrithione (ZPT) (µg/ml)	
MIC	MFC	MIC	MFC
16	64	0.5	4

Weckesser *et al.* (2007) reported that MIC of supercritical CO_2-extract from *Usnea barbata* against *M. furfur* HK001 was 10 µg/ml, whereas in this study the MIC of MEU against *M. furfur* ATCC 14521 was 16 µg/ml. The differences were because of different of yeast strain (ATCC 14521, HK001), species of *Usnea*, and extraction method.

Moreover, Lee & Lee (2010) reported that 17 plant essential including garlic (Allium sativum L.), caraway seed (*Carum calvi* L.), coriander her (*Coriandrum sativum* L.), balsam peru (Myroxylon balsamum var. pereirae Royle), catnip (Nepeta cataria L., oregano (*Origanum vulgare* L.), pepnyroyal (*Mentha pulegium* L.), savory (*Satureja hortensi* L.), thyme (*Thymus vulgaris* L.), cinnamom leaf (*Cinnamomum zeylanicum* Blume), pimento berry (*Pimenta dioica* (L).

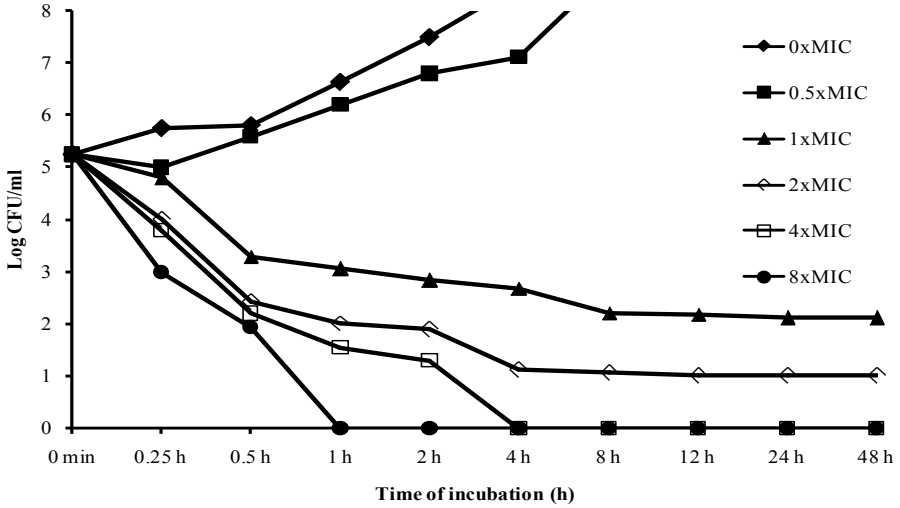

Figure 2. Time-kill plots M. furfur ATCC 14521 following exposure to methanolic extract of Usnea sp. (MEU) at 0×MIC (0 µg/ml) (filled diamonds), 1/2×MIC (8 µg/ml) (filled squares), 1×MIC (16 µg/ml) (filled triangles), 2×MIC (32 µg/ml) (open diamonds), 4×MIC (64 µg/ml) (open squares), and 8×MIC (128 µg/ml) (filled circles) after endpoint (48 h).

Merr.), clove bud (*Eugenia caryophyllata* Thumb.), clove leaf (*Eugenia caryophyllata* Thumb.), citronella java (*Cymbopogon nardus* L.), palmarosa (*Cymbopogon martini* Stapf.), lime (*Citrus aurantifolia* Swing.), and bay (*Pimenta racemosa* (Mill.) J.W. Moore) oils have possesses inhibitory activity against *M. furfur* at 2 mg/ml. Among them, oil of *Citrus auranifolia* was most active against *M. furfur* at 2 mg/ml with the diameter clear zone of 2.6 cm. In this study, 1 mg/ml of MEU resulted 3.4 m (34 mm) of diameter clear zone (Fig. 1B). This result indicated that antifungal activity of MEU against *M. furfur* is stronger than that of *Citrus auranifolia* oil.

In vitro MFC of MEU with endpoint after 48 h demonstrated that MEU was able to kill the *M. furfur* with MFC of 64 µg/ml. Time-kill curve (Fig. 2) demonstrated that MEU was able to kill 100% of *M. furfur* with 64 µg/ml for 4 h or 128 µg/ml for 1 h. MEU exhibits fast acting in killing of *M. furfur*, the cfu reduction was > 3log10 within 1 h.

CONCLUSION

These findings demonstrate that MEU has antifungal activity in vitro against. MEU may be potentially valuable as a natural agent for treating yeasts causing dandruff, tinea versicolor, seborrhoeic dermatitis and tinea capitis. However, this current study does not evaluate active compound(s) in the MEU. Further research will evaluate isolation of active compound(s) from MEU for antifungal agent against M. furfur.

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