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)

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 seborrhoeic 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 immunsuppressed 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 activity of Usnea sp. 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 developing 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 Harirhan, 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 CO2-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. furfur.

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 distilled 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 performed 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 5×10⁶ cfu/ml (a McFarland standard) by diluting 1:10 with SDHO medium to make final inoculum concentration of 5×10⁵ 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 gently. 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. furfur* 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 5×10⁶ cfu/ml was diluted 1:10 in SDHO or SDH medium to make final inoculum concentration of 5×10⁵ cfu/ml. Each concentration of MEU was diluted 1:10 in SDHO or SDH medium to make final inoculum concentration of 5×10⁵ cfu/ml. This procedure yielded an initial inoculum 4.5×
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 distilled 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 DISCUSSION

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. furfur* was 0.5 μg/ml and (Table 1). Thus, susceptibility of ZPT against *M. furfur* is in good agreement with previous report.

Weckesser et al. (2007) reported that MIC of supercritical CO₂-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.), corianderher (*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).
Merr.), clove bud (Eugenia caryophyllata Thumb.), clove leaf (Eugenia caryophyllata Thumb.), palmarosa (Cymbopogon martini Stapf.), lime (Citrus aurantifolia Swing.), and bay (Pimenta racemosa (Mill.) J.W. Moore) oils have possess inhibitory activity against M. furfur at 2 mg/ml. Among them, oil of Citrus aurantifolia 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 aurantifolia 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 M. furfur, 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.

**REFERENCES**


Anti Fungal Activity of Methanolic Extract of Usnea sp. against Malassezia furfur


