**Antimicrobial, Antioxidant, Hemolytic Activities and Toxicity of Ethyl Acetate Extract From an Unidentified Coral-Associated Fungus, *Aspergillus brevipes* RK06**

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## Abstract

Marine fungi are one of the potential and prolific sources to produce unique and novel structure of bioactive compounds. The aim of this research was to explore the biological activities potency from ethyl acetate extract of *Aspergillus brevipes* RK06. *A*. *brevipes* RK06 was successfully isolated from an unidentified coral from Randayan Island, Kalimantan Barat. The extract inhibited the oxidation of linoleic acid (Ferric thiocyanate assay) with a lipid peroxidation inhibition value of 28.44%. The IC50 value of the extract for brine shrimp lethality test was 34.19ug/mL. The hemolytic percentage of the extract for hemolysis on cow erythrocytes was 5.21%. The extract showed a growth inhibition against *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Based on the assays, the extract showed a potential citotoxity and both low antioxidant and hemolytic activities.

**Keywords:** antioxidant, hemolytic activity, antimicrobial, toxicity, *Aspergillus brevipes* RK06

**INTRODUCTION**

Exploration of novel compounds with great potential as pharmaceutical, nutritional supplements, cosmetics, agrichemicals and enzymes from many marine microorganism has been continuously done due to each of these marine byproduct has strong potential market value. In recent years, exploration of various sources to get natural antioxidant compounds has been an increasing interest because of widely application and safer. Antioxidant can be used as to prevent cardiovascular disease, stroke, and cancer. Antioxidant compounds can be also used to food additives such as to prevent lipid peroxidation lipid in oil and cosmetic such as anti aging. Issue multidrug resistant antibiotic and no effective anti cancer have been also trigger exploration antimicrobial and cytotoxic compounds.

Marine fungi are an important source of significant chemical diversity and pharmacologically active metabolites although many marine fungi are normally considered as terrestrial fungi. The Differences between marine fungi and terrestrial fungi in unique and unusual metabolite production are the influence on their environment especially adaptation of environmental pressures. For example, hirsutanols are biosynthetically related to several compounds reported from the terrestrial fungus *Coriolus consors* but a seawater-based culture of *C*. *consors* can yields new metabolites (Bugni and Ireland, 2004).

The fungus can produce many secondary metabolites and various biological activities such as antimicrobial, antioxidant. Therefore, the aim of this research is to explore the biological activities potency from ethyl acetate extract of *Aspergillus brevipes* RK06 especially antimicrobial, antioxidant, hemolytic and toxicity.

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**MATERIALS AND METHODS**

**Fungal Isolate**

Fungal isolate was successfully isolated from an unidentified soft coral collected on Mei 31st, 2008 from Randayan Island, Kalimantan Barat, Indonesia (Fig. 1). The fungal isolate was preliminary identified as *Aspergillus brevipes* RK06 based on colony morphology and conidiospore.

**Microorganism Test**

Microorganism tests were used to evaluate antimicrobial activity namely *Bacillus* sp., *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* *Eschericia coli*, *Vibrio vara*, *Vibrio cholerae,* *Vibrio harveyi,* *Salmonella* sp. and *Aeromonas hydrophila*, *Klebsiella pneuminiae*, *Citrobacter freundii*, and fungi namely *Candida albicans*.

**Cultivation and Metabolite Extraction**

The fungal isolate in petridish was inoculated onto 300 mL of M2+ broth and incubated on a rotary shaker at 140 rpm at 30 oC. After 30 days, the culture was homogenized using a blender and extracted using ethyl acetate as solvent. The ethyl acetate extract was concentrated using a rotary evaporator.

**Antimicrobial Activity Assay**

Antimicrobial activity of the fungal extract was determined based on modified well-difusion agar methods (Valgas *et al*., 2007). Well with diameter of 6 mm was made in plate containing nutrient agar media using a punch then its media was spreaded with inoculum of bacterial test. Furthermore the well was filled in 500 μg/well of the fungal extract and incubated at 37 oC for 24 h. The extract having antimicrobial activity was signed with formation of inhibition zone around isolate then diameter of inhibition zone (measured from the edge of the colony to the edge of the clear zone) was recorded.This procedure was also determined antimicrobial activity using fungal test, *C*. *albicans* but the medium assay was potato dextrose agar (PDA).

**Toxicity Assay**

The fungal extract was determined for the toxicity effects using larvae (nauplii) of *Artemia salina* (Brine Shrimp) (Piccardi et al., 2000). The eggs were placed in an aerated bottle containing 33 g L-1 NaCl saline and natural lighting. After two days of hatching period at ambient temperature, 10 larvae of *A*. *salina* was mixed with the fungal extract with various concentration, namely 100, 75, 50, 25 and 12,5 µg/mL. The mixture was diluted with distilled water until 1 mL then incubated at ambient temperature for 24 hours. The amount total of survived larvae was counted under microscope and recorded. The data were analyzed by SPSS version 17 to determine LC50 values.

**Hemolytic Assay**

Hemolytic assay was conducted refer to Kang *et al.* (2009). Erythrocytes were isolated from blood of cow. One mL of the fresh blood supplemented with Alsever’s solution (pH 7,4) and washed with 19 mL of Phosphate Buffered Saline(PBS) (pH 7,4) and then centrifuged at 1,500 x g for 5 menit at 4oC. The erythrocyte pellet was washed again 2 times. Furthermore, 1 g of the erythrocyte pellet was suspended with 100 mL of PBS and 100 uL of the erythrocyte suspension was mixed 100 uL of PBS and 100 uL of the fungal extract (200 ug/mL) and incubated at 37 oC for 30 min. Then, the suspension was centrifuged at 1.500 x g at 4 oC for 5 min. Absorbance of supernatant was read by microplate reader at 415 nm. Sodium Dodecyl Sulphate(SDS) was used as positive control. Percentage of hemolytic activity was calculated as follow:

*Percentage of hemolytic activity* = 

**Antioxidant Assay**

Antioxidant assay was performed based on Ferric thiocyanate method (Lindsey, 2002). The fungal extract was mixed with 10 uL of sesame oil and 1 mL of ethanol then incubated in dark place at ambient temperature. After 24 h, the mixture was added 20 µL of FeCl2 0.014 M, 20 µl KSCN 30%. Antioxidant activity was monitored by microplate reader at 500 nm. Ascorbic acid was used as positive control. Percentage inhibition of linoleic acid oxidation was calculated as follows:





**Figure 1. An Unidentified Soft Coral**

**RESULTS** **AND DISCUSSION**

 *A*. *brevipes* RK06 extract showed antimicrobial activity against 3 of 13 microrganisms test namely *K. pneumoniae*; *P. aeruginosa*; and *S. aureus* (Table I.). Antimicrobial spectrum of the extract is categorized as narrow antimicrobial spectrum. However, antimicrobial compounds contained the extract interest to determine its structures due to the extract can inhibit growth *P. aeruginosa* that is resistant to 34 kind of antibiotic commercial, namely cephazoline, amicacyn, amoxylin, ampicillin, sulbactam, clavulanic acid, carbenicillin, ciprofloxacin, cephalotin, chloramfenicol, sulfonamide, colistin, erythromycin, gentamycin, neomycin, ceftriaxone, nitrofurantoin, azithromicyn, asam nalidixic, norfoloxacin, pipemidic acid, piperacillin, sulfamethoxazole, tetracyclin, tobramicyn, tircarcillin, vancomycin, lincomycin, clindamcyin, kanamycin, doxycycline, cefuroxime, pefioxacyn dan meronem. Therefore, there is possibility to get a novel antimicrobial structure.

**Table I. Antimicrobial activities of ethyl acetate extract from *A*. *brevipes* RK06**

|  |  |
| --- | --- |
| **Microorganism Tests** | **Average Diameter of Inhibition Zones (cm)** |
| *Bacillus* sp. | 0 |
| *B*. *subtilis* | 0 |
| *V*. *vara* | 0 |
| *V*. *cholerae* | 0 |
| *V*. *harveyii* | 0 |
| *Salmonella* sp. | 0 |
| *E*. *coli* | 0 |
| *C. freundii* | 0 |
| *K*. *pneumonia* | 0.65 |
| *A. hydrophila* | 0 |
| *P. aeruginosa* | 0.32 |
| *S*. *aureus* | 0.61 |
| *C*. *albicans* | 0 |

*Artemia salina* is an invertebrate model used to study ecotoxicology and general toxicology of chemicals and natural products (Favilla *et al*., 2006). In addition, it can be used to predict cytotoxicity due to many researchers reported a positive correlation between brine shrimp lethality and cytotoxicity. LCB50 values of *A*. *brevipes* RK06 extract was 34.19 µg/mL which was determined based on simple linear regression equation (Fig. 2). The value was classified as a toxic level. Crude extract with LC50 values of less than 30 µg/mL, 30-1,000 μg/mL and more than 1,000 μg/mL were classified as a very toxic, toxic and non-toxic respectively (Meyer *et al*.,1982).

**Figure 2. Effect of ethyl acetate extract against shrimp larvae mortality**

Hemolytic activity is one of screening tools used to evaluate the potential biosurfactant producer (Kiran *et al*., 2009). Biosurfactants or microbial surfactants are surface active amphiphilic molecules produced by a number of microorganisms. The ethyl acetate extract of *A*. *brevipes* RK06 showed 5.21±3.98 % of hemolytic activity which can be categorized as a low hemolytic activity. A high and low hemolytic activity of extract is categorized with percentage of hemolytic activity more than 40% and 5-10 % respectively (Sperandio *et al.,* 2010). An antioxidant is any substances in low concentration significantly inhibit or prevent oxidation of the substrate. Ferric thiocyanate is a method used to inhibit oxidation of unsaturated fatty acid. An antioxidant compound that ability to inhibit this process is important due to many pathological events resulted from lipid peroxidation (Kosem *et al*., 2007). In addition, the method is simple and cheap and reproducible (Moon and Shibamoto, 2009). The *A*. *brevipes* RK06 extract (500 µg/mL) can inhibit lipid peroxidation 28.44±1.47%

**CONCLUSION**

The ethyl acetate extract of *Aspergillus brevipes* RK06 showed growth inhibition against *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Based on the assays, the extract showed a potential citotoxity and both low antioxidant and hemolytic activities.

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