Identification of a New Compound as α-Glucosidase Inhibitor from *Aspergillus aculeatus*

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Abstract

Terestrial fungi are of great importance as potential sources of pharmaceutical agent. *Aspergillus aculeatus,* a fungus isolated from soil sample collected in Indonesia, was cultured in liquid media to investigate a novel compound as inhibitor α-glucosidase. The mycelium extract of *A. aculeatus* shows potential activity against *Saccharomyces cereviseae* α-glucosidase and mild activity against mammalian α-glucosidase with IC_{50} values of 9.57 µg/mL and 470.76 mg/mL, respectively. Enzyme assay-guided fractionation of this extract afforded rubrofusarin (1). Rubrofusarin, a linear naphtho-γ-pyrone, is a natural pigment from *Aspergillus* sp. Interestingly, compound 1 shows potential inhibitory activity against mammalian α-glucosidase (IC_{50} of 92.7 µg/mL), but no to *S. cereviseae* α-glucosidase. The results suggest that *A. aculeatus* is a promising natural source as a lead compound in the discovery of antidiabetic drug.

Keywords: *Aspergisllus aculeatus,* α-glucosidase inhibitory activity, rubrofusarin

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Introduction

Microorganisms comprise of metabolites that are useful as antibiotics, or have been used as lead compounds for the development of pharmaceutical agent. Fungal metabolites have also been used as lead compound in other fields of medicinal chemistry (Marinelli, 2009). The genus of *Aspergillus* represents a diverse group of fungi that are the most abundant fungi in the world (Krijgsheld *et al*., 2012). *Aspergillus* is a filamentous, cosmopolitan and ubiquitous fungus commonly found in soil, plant debris and indoor air environment. The genus of *Aspergillus* includes over 185 species and it's famous for the production of bioactive secondary metabolites (*eg*. antibiotic, mycotoxin, antifungal compounds, *etc*) (Balajee, 2009). Recently, we reported that secondary metabolites isolated from ethyl acetate extract of *Aspergillus terreus*, such as sulochrin, butyrolactone I, and aspulvinone E showed potential inhibitory activity against α-glucosidase (Dewi *et al*., 2009; 2015).

Glycosidases are well known targets in the design and development of antidiabetic, antiviral, antibacterial, and anticancer agents (Du *et al*., 2006; Kim *et al.,* 2008). α-Glucosidase inhibitors competitively bind to the carbohydrate-binding region of α-glucosidase enzymes, and thereby compete with oligosaccharides to prevent their cleavage into absorbable monosaccharides (Baron, 1998; Cheng & Josse, 2004). In type II diabetic patients, delaying glucose absorption after meal by inhibtion of α-glucosidase is known to be beneficial during therapy.

In the present study we describe the fermentation, isolation, and characterization of α-glucosidase inhibitor compound isolated from *A. aculeatus.*

Materials and Methods

General. Melting points were measured on a Fisher Scientific melting point apparatus and were uncorrected. UV-Vis absorption spectra of the active compound in methanol were

recorded on a Agilent Technology Carry 60 spectrophotometer. The mass spectra were measured with LC-MS Mariner. The nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a JEOL JNM-ECA 500 with TMS as the internal standard. HMQC (Heteronuclear Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) techniques were used to assign correlations between ^{1}H and ^{13}C signals. The chemical shift values (δ) are given in parts per million (ppm), and coupling constant (*J*) in Hz. TLC was run on silica gel 60 F_{254} pre-coated plates (Merck 5554) and spots were detected by UV light.

Microorganisms and Culture Conditions. The fungus *A. aculeatus* LS04-3 was isolated from the Teluk Kodek area, Pemenang, West Lombok, Indonesia. It was identified as *Aspergillus aculeatus* NZD-mf39, according to its morphological characteristics and ITS rDNA sequence by Indonesian Culture Collection (InaCC), Indonesian institute of Sciences (LIPI). The fungus has been deposited in the InaCC and Research Center for Chemistry, LIPI. It was prepared on potato dextrose agar plates and stored at 4 °C, whereas working stock was grown on PDA at 25 °C for 7 days in the dark.

The growth medium for *A. aculeatus* LS04-3 was prepared by mixing 80 g of glucose and fresh potato extract (500 mL) in 3.5 L distilled water. The fresh potato extract was prepared by cutting 1 kg potatoes into small cubes and boiled with 2 L of distilled water, for 30 min. The obtained medium thus distributed among 80 flasks of 350 mL capacity (50 mL in each) and autoclaved at 121 °C for 30 min. Sample of seven days fresh mycelium of the fungus grown on PDA medium with in a petri dish at 28 °C were inoculated into the flask. After 10 days incubation under static conditions (25 °C), the culture broth was filtered through filter paper to separate the filtrate and mycelia. The mycelium was subjected to shaker for shaking with ethyl acetate (EtOAc) at 250 rpm for 20 minutes at room temperature. The extract was filtered and concentrated with a rotary evaporator under vacuum at 40 °C to obtain a brown solid (2 g).

Extraction and Isolation. The EtOAc extract (1.8 g) was chromatographed on silica gel (35×30 mm i.d) using a stepwise gradient from hexane (90 %) in EtOAc, to EtOAc (100 %) and then EtOAc (30 %) in methanol (MeOH) to obtain eleven fractions (F1-F11) based on TLC spot profiles. α-Glucosidase inhibitory activity showed that fraction 6, 10, and 11 were active against yeast α-glucosidase with an IC_{50} of 79.98, 54.43, and 157.09 µg/mL, respectively. However, those fractions showed weak inhibitory activity against mamalian α-glucosidase. Furthermore, fraction 4 showed activity against mamalian α-glucosidase (IC_{50} 94.71 µg/mL), but weak activity against yeast α-glucosidase. Further purification of fraction 4 by recrystalization with hexane and MeOH afforded compound 1 (10 mg).

Yeast α-Glucosidase Inhibition Assay. The inhibitory activity for α-glucosidase was assessed as reported by Kim *et al.* (2004) with minor modifications. The reaction mixture contained 250 µL of 3 mM *p*-NPG and 495 µL of 100 mM phosphate buffer (pH 7.0) added to a tube containing 5 µL of sample dissolved in DMSO at various concentrations (5 to 100 µg/mL). The reaction mixture was pre-incubated for 5 min at 37 °C, the reaction was started by addition of 250 µL of α-glucosidase (0.065 units/mL) and the incubation was continued for 15 minutes. The reaction was stopped by addition of 1 mL of 0.2 M Na_2CO_3. The inhibitory effect on α-glucosidase activity was determined by measuring the amount of *p*-nitrophenol released at λ 400 nm. Individual blanks for the test samples were prepared to correct background absorbance where the enzyme was replaced with 250 µL of phosphate buffer. All the test were run in triplicate.

Mammalian α-Glucosidase Inhibition Assay. The inhibitory activity toward mammalian α-glucosidase was assayed as described by Sancheti *et al*. (2011) with a slight modification: 0.5 grams of rat small-intestinal acetone powder was suspended in 10 mL of 0.9 % saline (100:1 w/v), and the suspension was sonicated twelve times for 30 seconds at 4 °C (properly). After centrifugation (1,000 g, 30 min, 4 °C), the supernatant was used for the assay. Five microlitres of sample solution (50-200 µg/mL)

was pre-incubated with 595 µL of 0.1 M phosphate buffer (pH 7.0), and 250 µL of 5 mM p-NPG solution in 0.1 M phosphate buffer (pH 7.0). After pre-incubation at 37°C for 5 min, 150 µL of mammalian α-glucosidase solution was added. The reaction was then terminated by the addition of 1mL of 0.2 M Na_2CO_3. Absorbance of the tested sample was recorded at 400 nm. Individual blanks for test samples were prepared to correct background absorbance, where the enzyme was replaced with 150 µL of phosphate buffer. All the tests were run in triplicate.

Results and Discussion

Interest in the isolation of α-glucosidase inhibitors from certain microorganisms has increased due to fast growing characteristic of microorganisms. In the present study, the antidiabetic activity of the extract was evaluated using α-glucosidase from two different sources; yeast and mammalian.

A yeast α-glucosidase enzyme from *S. cereviseae,* which categorized as α-glucosidase type I, was selected for general screening of α-glucosidase inhibitors. This study used quercetin and acarbose as references. Due to several reports that quercetin as a phenolic compound have stronger inhibitory activity on α-glucosidase from yeast *S. cerevisiae* than acarbose (Tadera *et al.,* 2006), whereas voglibose and acarbose have high inhibitory effects on mammalian α-glucosidase, but no inhibitory activity against yeast *S. cerevisiae* (Kim *et al*., 2004). As reported previously, α-glucosidase broadly consists of type I from yeast *S. cerevisiae* and type II from the mammalian species, and there are structural differences between these types (Kim *et al.,* 2005).

Mycelium extracts of *A. aculeatus* was found to inhibit yeast α-glucosidase with an IC_{50} value of 9.57 µg/mL, while the filtrate extract showed no significant activity. The mycelium extract (2 g) of *A. aculeatus* was further purified to isolate the secondary metabolite compounds with inhibitory activity against α-glucosidase using silica gel chromatography separation yielded eleven fractions (F1-F11). Each collected fraction were tested for α-glucosidase inhbitory activity (Table 1).

Based on the results (Table 1), fraction 6, 10, and 11 are confirmed to have inhibitory activity against yeast α-glucosidase with IC_{50} values of 79.88, 11.05, and 78.67 µg/mL, respectively. However, those fractions weak against mammalian α-glucosidase. On the other hand, the fraction 4 show significant activity against mammalian α-glucosidase (IC_{50}= 92.70 µg/mL) but exhibit low inhibitory activity against yeast α-glucosidase (IC_{50} of > 1000 µg/mL). The inhibitory activity of F-4 has a similar pattern with acarbose, which actively inhibit mammals α-glucosidase but not in yeast α-glucosidase.

Table 1. Yield and α-glucosidase inhibitory activities of chromatography fraction

Sample	Yield (mg)	Inhibitory activityof α-glucosidase (%)	
		Yeast*	Mammalian*
F1	2.2	nd	nd
F2	39.4	nd	nd
F3	23.5	nd	nd
F4 (**1**)	15.0	13.94±2.1	52.9±3.4
F5	35.5	nd	nd
F6	202.9	69.18±0.7	41.09±2.3
F7	34.5	nd	nd
F8	15.6	nd	nd
F9	33.6	nd	nd
F10	328	98.06±3.1	2.96±2.9
F11	343.7	67.69±2.0	nd
Quercetin	-	74.72±1.2	23.25±4.2
Acarbose	-	14.04±3.2	68.44±3.2

Data are presented as the mean ± S.D. of triplicate measurements
*α-Glucosidase inhibitory activity determinant at concentration 100 µg/mL of samples
nd: not determined

Fraction 6, 10, and 11 were further purified by silica gel-column, sephadex, and preparative TLC, unfortunately we failed to get a pure single compound. Further purification of fraction 4 by recrytallization with *n*-hexane and methanol afforded orange solid substance (1).

Compound 1 was obtained as orange solid, mp: >250 ºC, showed a molecular weight *m/z* 273.343 (LCMS), correspond to the molecular formula $C_{15}H_{12}O_5$ $[M+H]^+$. The UV spectrum (in methanol) showed maximum absorbance (λ_{max}) at 406, 277, 252 nm. The FTIR transmittance band at 3400 $cm^{-1}$for hydroxyl

group (–OH) and 1610 $cm^{-1}$for carbonyl (C=O).

The ^{1}H NMR spectra (Table 2) displays a chelated hydroxyl group (δ_H 14.91), two *m*-coupled doublet signals (δ_H 6.47, 6.70) and one singlet signal (δ_H 6.04), along with one methoxy signal (δ_H 3.92) and an sp^2 linked methyl (δ_H 2.38).

Table 2. ^{1}H and ^{13}C NMR data of rubrofusarin (Leeper & Staunton, 1984) and compound 1.

Number	Rubrofusarin ($CDCl_3$)		Compound **1** (Acetone-*d6*)	
	δ_H	δ_C	δ_H	δ_C
2	-	163.7		169.1
2-CH_3	2.28 (3H,s)	19.68	2.38	20.5
3	5.95 (1H,s)	110.5	6.04 (1H,s)	107.6
4	-	177.8	-	185.1
4a	-	113.9	-	104.3
5	-	159.6	-	162.3
5a	-	114.4	14.9	154.1
6	-	159.6	-	160.9
7	6.42 (1H, d, *J* 2Hz)	98.9	6.44 (1H, d, *J* 2Hz)	100.7
8	-	160.6	-	164.2
8-OCH_3	-	55.2	-	55.4
9	6.61 (1H, d, *J* 2Hz)	97.5	6.69 (1H, d, *J* 2Hz)	102.0
9a	-	139.8	-	142.4
10	7.34 (1H,s)	108.3	6.93 (1H,s)	97.96
10a	-	154.75	-	159.0

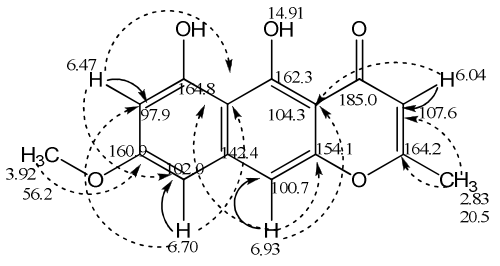
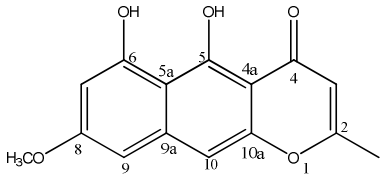


Figure 1. HMQC (—) and HMBC(---) correlations of compound 1.

The ^{13}C/HMQC spectra (Figure 1) indicated the presence of 15 carbon signals, including 4 sp^2 methines (δ_C 107.5-97.9), 3 sp^2-oxy carbons (δ_C 162.3-160.9), 1 carbonyl of γ–lactone (δ 185.0) (Leeper & Staunton, 1984; Shaaban *et al*., 2012), 5 non-oxygenated sp^2, 1 aromatic attached methyl ethers (δ_C 56.2) and 1 sp^2-attached methyl (δ_C 20.5). Characterization of the active compound by spectroscopic methods, revealed and conferred with previous report (Stout, 1962; Leeper & Staunton, 1984), confirm that compound 1 to be fully consistent with 5,6-Dihydroxy-8-methoxy-2-methyl-4*H*-naphtha [2,3-b]pyran-4-one) or rubrofusarin (Figure 2).



5,6-dihydroxy-8-methoxy-2-methyl-4*H*-benzo[*g*]chromen-4-one

Figure 2. Chemical structure of rubrofusarin (compound 1).

Rubrofusarin is an orange crystalline substance which was first isolated from the mycelium of strain of the mold *Fusarium culmorum* (Stout, 1962), and produced by a wide range of different fungal species, such as *Fusarium graminearum*, *Aspergillus niger*, *Aspergillus parasiticum*, and *Ustilaginoidea virens* (Rubgbjerg *et al*., 2013). The biological activity of rubrofusarin was reported to include inhibition of human DNA topoisomerase II-α, and general antibiotic effect on Mycobacterium tuberculosis and various filamentous fungi (Rubgbjerg *et al*., 2013). Additonally, rubrofusarin exhibited moderate tyrosinase inhibitory activity, with an IC_{50} value of 65.6 µM (Huang *et al*., 2010). However, rubrofusarin as α-glucosidase inhibitor has never been reported.

Agents with a-glucosidase inhibitory activity have been useful as oral anti hypoglycemic agents for the control of hyperglycemia in patients with diabetes (Christhudas *et al*., 2013). In the present study, the inhibitory activity of compound 1 was evaluated using *S. cereviceae* and mammalian α-glucosidase with Quercetin and Acarbose as reference compounds. Compound 1 and acarbose show no inhibitory activity against yeast α-glucosidase (IC_{50}>200 µg/mL), while quercetin show high activity with an IC_{50} of 23.75 µg/mL. Interestingly, compound 1 shows potential inhibitory activity against mammalian α-glucosidase (IC_{50} of 92.70 µg/mL) while it is compared to quercetin (IC_{50} of 208.39 µg/mL), however lower than Acarbose (IC_{50} of 67.93 µg/mL).

Conclusions

In conclusion, the present study reveals that rubrofusarin, a secondary metabolite isolated from *A. aculeatus,* is a potential antidiabetic exhibiting inhibitory activity against mammalian α-glucosidase with an IC_{50} of 92.70 µg/mL. The results suggest that *A. aculeatus* is a promising natural source for discovery lead compound of antidiabetic. Further investigations to explore the active compounds from *A. tubingensis* are underway in our laboratories.

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