α-GLUCOSIDASE INHIBITORY EFFECT OF SULOCHRIN FROM *ASPERGILLUSTERREUS* AND ITSBROMINATED DERIVATIVES

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Abstract Use of α-glucosidase inhibitorsis one of the therapeutic approaches for decreasing postprandial hyperglycemia. Sulochrin (**1**) from *Aspergillus terreus* as well as two synthetic sulochrin derivatives were assessed for antidiabetic activity against yeast and rat intestine α-glucosidase. Sulochrin showed potential inhibition against yeast α-glucosidase, through a non-competitive mode with an IC_{50} value of 133.79 µM, and rat intestine α-glucosidase by uncompetitive mode with an IC_{50} value of 144.59 µM.. Two synthetic derivatives of sulochrin were also prepared by bromination which resulted in dibromo-sulochrin (**2**) and tribromo-sulochrin (**3**). Preliminary SAR studies of sulochrin derivatives revealed that the yeast α-glucosidase inhibitory activity of compound **2** and **3** increased than **1** due to substitution of hydrogen atom with bromine with IC_{50} values of 122.65 and 49.08 µM, respectively. However, the inhibitory activity against rat intestine α-glucosidase of **2** and **3** was decreased compared to **1.** To the best our knowledge, this is the first report of structure-activity relationship of sulochrin and its derivatives as α-glucosidase inhibitors. These results suggested that sulochrin can potentially be used as a lead compound to develop new α-glucosidase inhibitor from microorganisms.

Keywords: *Aspergillus terreus*, α-glucosidase inhibitory activity, sulochrin, bromination

INTRODUCTION

Type-2 diabetes is the most common form of diabetes, accounting for 90% of cases, and is usually characterized by an abnormal rise in blood sugar right after a meal, called postprandial hyperglycemia(Nguyen *et al.,* 2011). α-Glucosidase inhibitorsare one of the therapeutic approaches in decreasing postprandial hyperglycemia by delaying the digestion of poly-and oligosaccharides to absorbable monosacharides (Takahashi & Miyazawa, 2012). Furthermore, glucosidase inhibitors have been a huge concern to researchers working in the field of medicinal chemistry since their antidiabetic and antiobesity activity, is also associated with their activity against HIV and hepatitis (Mehta *et al.,* 1998; Du *et al.,* 2006; Zhu *et al.,* 2008). Acarbose is a compound isolated from *Actinoplanes utahanensis* that has been attributed with a

decrease of postprandial blood glucose and used as medicine for treating patients with type 2diabetes (Fujisawa *et al.,* 2005; Kim *et al.,* 2005). However, long-term administration of acarbose has been associated with diarrhea, abdominal gas, liver toxicity, and adverse gastrointestinal symptoms that would increase the risks of liver diseases (Kim *et al.,* 2008). Therefore, research on the exploration of new α-glucosidase inhibitor for further drug development is still necessary.

Aspergillus terreus is a common soil saprophyte was isolated from both marine and terrestrial sources with worldwide distribution and its studies were first published in 1918 (Wang *et al.,* 2008). A statin drug, lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase is known for its cholesterol-lowering effect used in the treatment of heart disease and atherosclerosis, is mainly produced by *A. terreus* (Hajaj *et al.,* 2001). Recently, we have reported that butyrolactone I and aspulvinone E isolated from an ethyl acetate extract of *A. terreus* showed potential inhibitory activity against α-glucosidase (Dewi *et al.,* 2014; Dewi *et al.,* 2015). As part of our research program on the utilization of bioresources in Indonesia, we have been screening extracts of *A. terreus* for *in vitro* antidiabetic activity, namely for α-glucosidase inhibitory activity. In one of our previous study, extract of *A. terreus* exhibited a significant inhibitory effect against yeast α-glucosidase and suppressed postprandial hyperglycemia in mice (Dewi *et al.,* 2007). Therefore, we selected the strain for large scale fermentation with a view to isolate the active compound and study the effect on bioactivity upon bromination. The effect of isolated and derived compounds was evaluated on yeast and mammalian α-glucosidase by *in vitro* assay. The structure-activity relationships and kinetics of inhibitory activity of active compounds were also discussed.

MATERIALS AND METHODS

General instrumentsand reagents

Melting points were measured on a Yanaco micro melting point apparatus (Yanaco Co., Ltd., Kyoto, Japan) and were uncorrected. The mass spectra of the compounds were measured with Mariner Liquid Chromatography - Mass Spectrometer. 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 using CD_3OD as solvent, with TMS as internal standard. HMQC and HMBC 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. Chromatography column was carried out using Merck Si-gel 60 and TLC analysis on pre-coated Si-gel plates (Merck Kieselgel 60 F_{254}) and spots were detected under UV light. All solvents used were analytical grade and distilled prior to use.

α-Glucosidase Type I: from yeast *Saccharomyces cerevisiae* (EC 3.2.1.20), bovine serum albumin and *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) as synthetic substrate of α-glucosidase were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). α-Glucosidase Type II rat intestinal acetone powder (as source of

crude intestinal α-glucosidase), benzyltrimethylammoniumtri-bromide (BTMAT-Br), and corn step liquor were obtained from Sigma-Aldrich.

Fungal material

A. terreus, a mutant developed from ATCC 20542, was obtained from the Research Center for Chemistry, Indonesian Institute of Sciences (RCC-LIPI), Indonesia. The voucher specimen was deposited at RCC-LIPI at -20°C, whereas working stocks were prepared on MY agar (malt extract 1%, yeast extract 0.4%, dextrose 0.4%, and agar 2%) stored at 4°C.

Extraction and isolation

The solid state fermentation was done as described in our previous report (Dewi*et al*., 2007). The solid state fermentation (30 Kg) product was extracted with EtOAc (15 L). The EtOAc extract was concentrated to obtain brown paste (500 g). After the addition of water:MeOH (4:1) (500 mL), the solution was partitioned with *n*-hexane (1 L), CH_2Cl_2 (5 L), and EtOAc (3 L) successively and concentrated subsequently. Organic fractions were subjected to primary α-glucosidase inhibitory activity screening, and EtOAc fraction was found to be the most active (Fig. 1). The EtOAc fraction (25 g) was applied to a silica gel column chromatography (CC) eluted with a linear gradient concentration of *n*-hexane/EtOAc from 10% to 100% to obtain ten fractions (F1-F10). Fraction 6 (6.5 g) was rechromatographed on a silica gel CC eluted using a stepwise gradient from 70% *n*-hexane in EtOAc to 100% EtOAc to give eight fractions (F6.1-F6.8). Further separation of fraction F6.5 by chromatography on a silica gel column, eluted with isocratic elution of $CHCl_3$:MeOH (3:1) followed by recrystallization from MeOH yielded yellowish solid compound **1** (3 g).

Preparation of sulochrinderivatives

Derivatization of sulochrin (**1**) was conducted by bromination and cyclization. Bromination of **1** was carried out according to the method of Sato *et al*.(2005). Compound **1** (40 mg, 0.12 mmol) was dissolved in $CHCl_3$/MeOH (1 mL), mixed with $CaCO_3$ (25 mg, 0.25 mmol) and benzyltrimethylammoniumtribromide (BTMAT-Br) (95 mg, 0.24 mmol). After 30 min of stirring at room temperature, the reaction mixture was added with $CHCl_3$, and washed with 5% HCl. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by recrystalization from $CHCl_3$ and MeOH, generating dibromo-sulochrin (**2**) (22 mg, 37%) as yellowish solid and tribromo-sulochrin (**3**) (29 mg, 42.5%) as yellow needle, where the substitution occurs on atoms C-3 and C-5 fordibromo-sulochrin (**2**), and atoms C-3, C-5 and C-6’ for tribromo-sulochrin (**3**).

Characterization of the isolated compound and its brominated derivatives

***Compound (1):*Sulochrin:** Methyl2-(2’,6’-dihydroxy-4’-methylbenzyl-)5-hydroxy-3-methoxybenzoate), yellow solid; mp 262℃; UVλ_{max}^{MeOH} 283 nm. FTIR (KBr) V_{max} 3358, 3086, 2918, 1691, 1593cm^{-1}. Molecular formula $C_{17}H_{16}O_7$ (m/z 333.08 $[M+H]^+$).^{1}H-NMR (500 MHz, CD_3OD) δ:2.18 (3H,s, H-11), 3.65 (3H,s, H-1), 3.68 (3H,s, H-10), 6.09 (2H,s, H-3’/5’), 6.64 (1H, d, *J*=2 Hz, H-4), 6.93 (1H, d, *J*=2Hz, H-6). ^{13}C-NMR (125

MHz,CD_3OD) δ:20.7 (C-11), 51.2 (C-9), 55.2 (C-10), 102.9 (C-4), 107.4 (2C, C-3'/5'), 109.5 (C-6), 110.5 (C-1'), 126.7 (C-2), 128.5 (C-1), 147.9 (C-4'), 157.3 (C-3), 158.3 (C-4), 162.0 (2C, C-6'/2'), 166.7 (C-8), and 200.7 (C-7).

Compound (2): **Dibromo-sulochrin:** Methyl 2-(3',5'-dibromo-2',6'-dihydroxy-4'-methylbenzyl-)5-hydroxy-3-methoxybenzoate), yellowish solid, mp 197-199℃; UV λ_{max}^{MeOH} 270 and 353 nm. FTIR (KBr)V_{max} 3390, 2924, 1691, 1579, 659cm^{-1} . Molecular formula $C_{17}H_{14}Br_2O_7$ (m/z488.08 $[M+H]^+$).^{1}H-NMR (500 MHz, CD_3OD) δ:2.18 (3H,s, H-11), 3.65 (3H,s, H-9),3.68 (3H,s, H-10), 6.64 (1H, d, *J*=2 Hz, H-4), 6.93 (1H, d, *J*=2Hz, H-6).^{13}C-NMR (125 MHz,CD_3OD) δ:25.7 (C-11), 53.1 (C-9), 56.5 (C-10), 101.1 (2C, C-3'/5'), 104.8 (C-4), 109.9 (C-6), 125.9 (C-2), 132.6 (C-1), 145.9 (C-4'), 155.0 (C-3), 155.8 (2C, C-2'/6'), 157.5 (C-5), 166.1 (C-8), and 196.8 (C-7).

Compound (3): **Tribromo-sulochrin:** Methyl 2-bromo-6-(3',5'-dibromo-2',6'-dihydroxy-4'-methylbenzoyl-)5-hydroxy-3-methoxybenzoate), yellow needles, mp 196-198℃; UVλ_{max}^{MeOH} 288, 355 nm. FTIR (KBr)V_{max} 3296, 2933, 1689, 1579, 626cm^{-1} . Molecular formula $C_{17}H_{13}Br_3O_7$ (m/z 566.54 $[M+H]^+$).^{1}H-NMR (500 MHz, CD_3OD)δ:2.18 (3H,s, C-11), 3.65 (3H,s, H-9), 3.68 (3H,s, H-10), 6.64 (1H, d, *J*=2 Hz, H-4).^{13}C-NMR (125 MHz,CD_3OD) δ:25.7 (C-11), 49.3 (C-9, 49.4 (C-10), 101.7 (2C, C-3'/5'), 104.9 (C-4), 113.1 (C-6), 125.3 (C-2), 136.2 (C-1), 146.5 (C-4'), 157.7 (C-3), 158.7 (C-5), 159.1 (2C, C-2'/6'), 168.7 (C-8), and 198.4 (C-1).

Yeast α-glucosidase inhibitoryassay

α-Glucosidase inhibitory activity was evaluated according to thepreviously reported method (Dewi *et al.*, 2014). 250 µL α-Glucosidase (0.124 unit/mL), 495 µL of 0.1 M phosphate buffer (pH 7.0), and 5 µL of various concentrations of samples in DMSO (50-200 µg/mL) were pre-incubated at 37°C for 5 min. The reaction was initiated/triggered by the addition of 250 µL of 5 mM *p*-NPG (Wako, Osaka, Japan). The reaction mixture was incubated at 37°C for 15 min and stopped by adding 1 mL of 0.1 M Na_2CO_3. α-Glucosidase activity was determined by measuring the release of *p*-NPG at 410 nm.

Mammalian α-glucosidase inhibitory assay

The inhibitory activity assay toward mammalian α-glucosidase was as described by Sanchetiet*et al*., (2011), with a slight modification, *i.e*: 0.5 grams of mammalian α-glucosidase (Sigma, St. Louis, MO, USA) was suspended in 10 mL of 0.9% saline (100:1 w/v), and the suspension was sonicated twelve times for 30s each time at 4°C (properly). After centrifugation (1000g, 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. The resulting absorbance wese recorded at 400 nm. Individual blanks for test samples were prepared to correct background absorbance, in which the enzyme was

replaced with 150 µL of phosphate buffer. All the tests were run in triplicate. The percent inhibition of α-glucosidase was assessed/determined using the following formula: % Inhibition = [1- (As/Ao)] ×100, where Ao is the absorbance of the control and As is the absorbance in presence of sample. The IC_{50} values were calculated from the mean inhibitory values by applying logarithmic regression analysis.

Kinetics of inhibition against α-glucosidase

The inhibitory activity of the active compounds against α-glucosidase activity was measured with increasing concentrations of *p*-NPG as a substrate in the absence or presence of anactive compound at different concentrations. The type of inhibition was determined by Lineweaver–Burk plot analysis

RESULTS AND DISCUSSION

A pilot-scale fermentation of *A. terreus*was made to obtain sufficient quantities of active components. After seven days of fermentation at 25°C, 500 g of EtOAc soluble material was extracted from 30 kg of the solid state (rice). Dried EtOAc extract (490 g) was sequentially partitioned with *n*-hexane, CH_2Cl_2 and EtOAc, respectively. The EtOAc fraction showed potential α-glucosidase inhibitory activity with the IC_{50} value of 8,6µg/mL. The further separation by column chromatography afforded sulochrin as an active compound of EtOAc fraction. The isolated scheme ofsulochrin is shown in Figure 1. Compound **1** was identified as (2-(2,6-Dihydroxy-4-methyl-benzoyl)-5-hydroxy-3-methoxy-benzoic acid methyl ester (sulochrin) by comparison of obtained spectra NMR data with published NMR data (Sato *et al.*, 2005; Ohashi *et al.*, 1997).

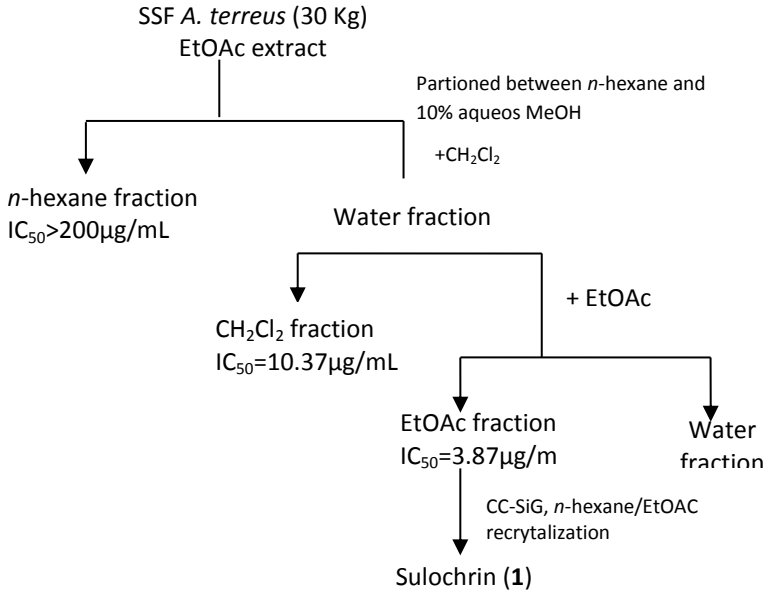

Figure. 1 Isolation procedure for sulochrin (**1**)

Sulochrin (**1**) has a unique benzophenone scaffold, in which the four ortho positions are all substituted. This compound is known as a metabolite of fungi and has very weak antibacterial and antifungal activities (Ueno *et al.*, 1998). Furthermore, Ohashi*et al*.(1997), have reported the inhibitory action on eosinophil degranulation by sulochrin and suggested it as a potentiallead for the development of newanti-allergic drug. However, the α-glucosidase inhibitory activity of sulochrin has never been reported before.

In order to confirm the structure, sulochrin was derivatized by bromination (Figure 2). The bromination of compound **1** with BTMAT-Br resulted in the substitution of a hydrogen atom with bromide. Semisynthetic derivatization was conducted to verify the effect on bioactivity upon brominationof sulochrin and to provide a preliminary study of the structure-activity relationship of sulochrin. Compound **2** showed cluster peaks at *m/z* 486, 488, and 490, which indicated that it was dibrominated, whereas compound **3** had cluster peaks at *m/z* 566, 568, 570, and 572, which suggested it was tribrominated (Kim *et al.*, 2008). The molecular weight of **2** and **3** was different by78 mass units which means compound **3** has undergone an extra Br substitution than compound **2**. A comparison of the ^{1}H and ^{13}C-NMR spectra of compound **1**, **2**, and **3** showed similar patterns. The significant differences can be observed from the substitution of aromatic protons by bromine in compound **1** to produce compounds **2** and **3**.

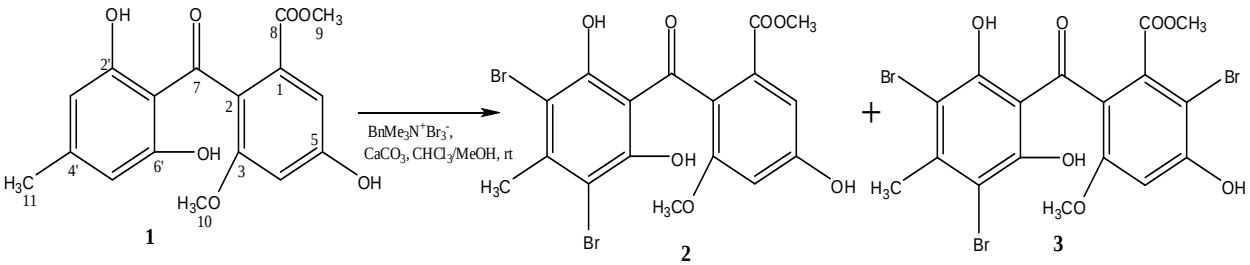

Figure.2 Derivatization of sulochrin (**1**)

α-Glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) is an exo-type carbohydrase that catalyzes the liberation of α-glucose from the nonreducing end of the substrate. Various types of α-glucosidases are widely distributed in microorganisms, plants, and animal tissues and the substrate specificity of α-glucosidases is known to differ greatly depending on their source (Kimura *et al.*, 2004). α-Glucosidase derived from bacterial, yeast (*Saccharomyces cerevisiae*) and insect enzymes, named α-glucosidase I, show higher activity toward

such heterogeneous substrates as sucrose and *p*-nitrophenyl α-glucoside (PNPG), whereas α-glucosidase from the mold, plant, and mammalian enzymes, named α-glucosidase II, hydrolyze the homogeneous substrates more rapidly than the heterogeneous substrates (Kimura et al., 2004). In order to evaluate the activity and selectivity of sulochrin and and its semisynthetic derivatives (**2** and **3**) for the inhibitory effect on α-glucosidase, yeast *S. cerevisiae* (I) and mammalian enzyme (II), were used. In this study, quercetinwas used as a standard due to several reports that quercetin, a phenolic compound, have stronger inhibitory activity on α-glucosidase from yeast *S. cerevisiae* than acarbose(Tadera *et al.,* 2006; Li *et al.*, 2009), while we have used acarbose as the standardagainst mammalian α-glucosidase.

α-Glucosidase inhibitory activity of the compounds (**1**-**3**) obtained were measured using spectrometric method at concentrations 25-250 µg/mL. For each compound, the IC_{50} value was calculated and presented in Table 1. The results indicated that the substitution of ahydrogen atoms in sulochrinbybromine in compounds **2** and **3** enhanced the inhibitory activity against yeast α-glucosidase. The IC_{50} values of compounds **2** and **3** against yeast α-glucosidase were 122.65 and 49.08 µM, respectively, which were lower than that of sulochrin. This finding is similar to that of Kim *et al*. (2008) which showed that two bromophenol isolated from *G. elliptica*: 2,4,6-tribromophenolresulted in a higher inhibitory activity than 2,4-dibromophenolagainst yeastα-glucosidase. Liu *et al*.(2011), also reported that the activity of bromophenol has a close relationship with the Br and phenolic unit in the molecule, for example, 3-bromo-4,5-dihydroxybenzyl alcohol inhibits against α-glucosidase with IC_{50} of 100 µM, when one more position is brominated, the IC_{50} value decreases to 89 µM.So it can be assumed that the inhibitory activity of bromophenol increases with the degree of bromo-substitution per benzene ring. To the best of our knowledge, this is the first report of identifying α-glucosidase inhibitory activity of sulochrin from *A. terreus*as well as dibromo- and tribromo derivative of sulochrin.

The inhibitory activity of active compounds against mammalian α-glucosidase were also compared with those of acarbose and quercetin (Table 1).The IC_{50} value of acarbose was 67.93 µM, whereas the IC_{50} value of quercetin was 108.39 µM. On the other hand, all compounds showed weak inhibitory activity against mammalian α-glucosidase,compared to that against yeast α-glucosidase, except for sulochrin. Sulochrin showed similar inhibitory activity against both yeast and mammalian glucosidase with the IC_{50} values being 133.79 and 144.59 µM, respectively (Table 1). These results supported our previous study where sulochrinwas able to bind at the active site ofα-glucosidase enzyme in molecular docking approach (Dewi *et al.*, 2009).

The mechanismfor the inhibitory activity of the isolated compounds wasanalyzed further using Lineweaver–Burk plots. The mode of inhibition of all active compounds againstyeastα-glucosidasewas analysed from the data derived from enzyme assays containing different concentrations of *p*-NPG, ranging from 0.25 to 5 mM of the compounds.

Table 1 α-Glucosidase inhibitory activities of sulochrin and its derivatives

Compound	**IC_{50} (μM)**[a]	
	Yeast α-glucosidase	**Mammalian α-glucosidase**
1	133.79±1.5	144.59±3.2
2	122.65±1.3	144.30±1.2
3	49.08±0.7	111.18±0.5
Quercetin	25.83±1.3	108.39±2.1
Acarbose	NI	67.93±1.3

a: The IC_{50} value was defined as the inhibitor concentration to inhibit 50% of α-glucosidase activity under assay conditions.
NI: no inhibition.

The 1/V increased with the concentration of compound **1**, but the *Km* remained constant (Figure 3a), suggesting non-competitive inhibition by compound **1**. While compound **2** and **3** displayed a mixed mode inhibition (Figure 4a and 5a). The mixed type of inhibition was characterized by a combination of competitive and non-competitive inhibition, which indicated that compound **2** and **3** bind to a site other than the active site of the enzyme and interact with either the free enzyme or the enzyme-substrate complex, possibly interfering with the actions of both (Mayur *et al.,* 2010). The inhibitory mechanism of those compounds was similar to that of quercetin (Tadera *et al.,* 2006; Li *et al.,* 2009) with *Ki* (inhibitory constant) value of 27.13 μM. The *Ki* values of compounds **1**, **2**, and **3** were 187.63, 131.25, and 117.20 mM, respectively (Table 2), which shows that compound **3** was the most effective in α-glucosidase inhibition.

The inhibitory mechanisms of all compounds (**1**-**3**) against mammalian α-glucosidase were shown in Figure 3b, 4b, and 5b. The results showed a straight line parallel to the plot of 1/V versus 1/[S] indicating uncompetitive inhibition. While acarbose exhibited competitive type inhibition with Ki value of 0.06 μM. These different inhibitory activities may be caused by structural differences, as previously reported, α-glucosidase broadly consists of type I from yeast *S. cerevisiae* and type II from the mammalian species, and there are homology analysis of the complete amino acid sequences differences between these types (Ueno *et al.,* 1998).

Table 2 Type and kinetic constant of yeast and mammalian α-glucosidase inhibition by sulochrin and its derivatives

Compond	Inhibition mode		Inhibition constans (*Ki*μM)	
	Yeast	mammalian α-glucosidase	Yeast	mammalian α-glucosidase
1	Non-compettive	Uncompetitive	187.63	85.42
2	Mixed type	Uncompetitive	131.25	94.69
3	Mixed type	Uncompetitive	117.20	104.20
Quercetin	Mixed type	nt	27.13	-
Acarbose	nt	Competitive	-	0.06

nt; not tested

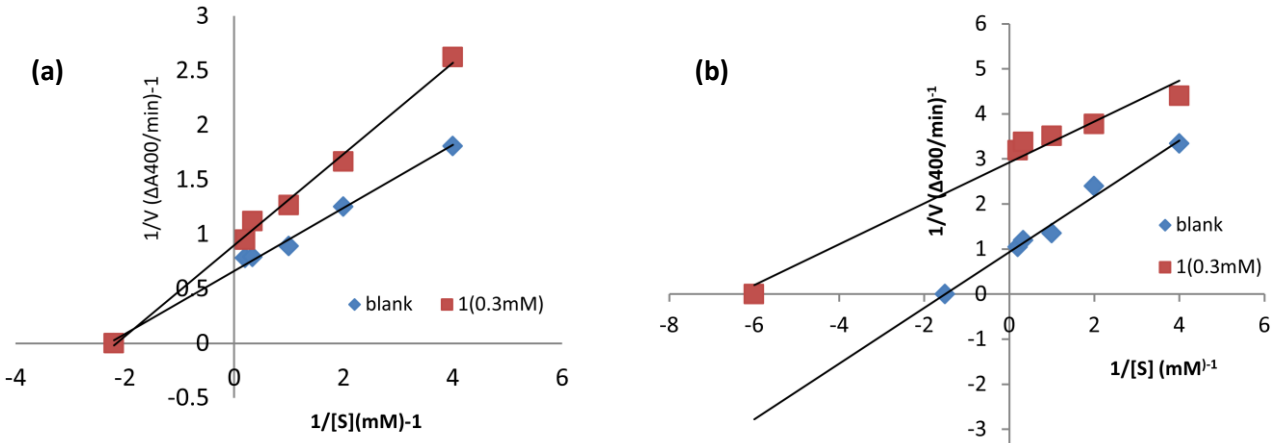

Figure.3 Lineweaver-Burke plots of compound **1** against yeast (a) and mammalianα-glucosidase (b)

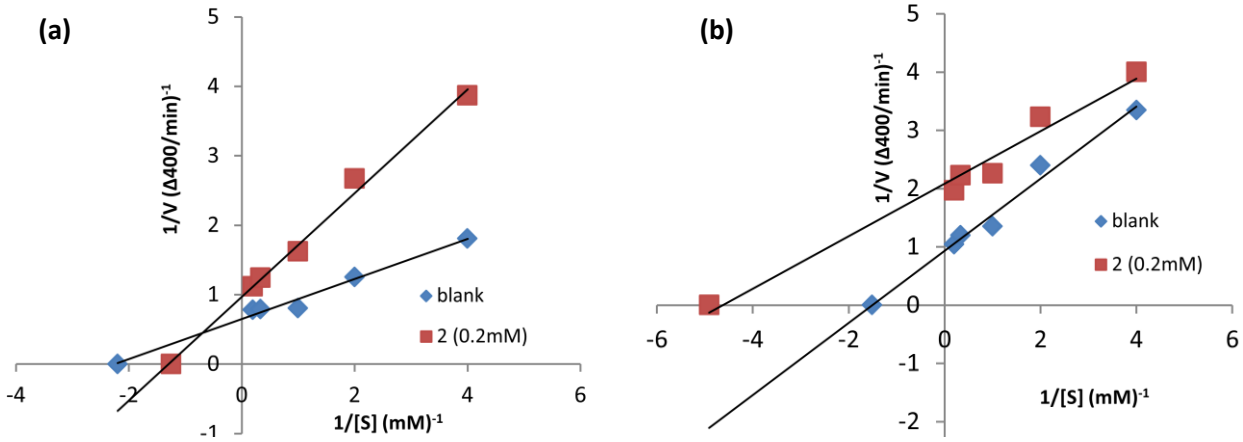

Figure.4 Lineweaver-Burke plots of compound **2** against yeast (a) and mammalianα-glucosidase (b)

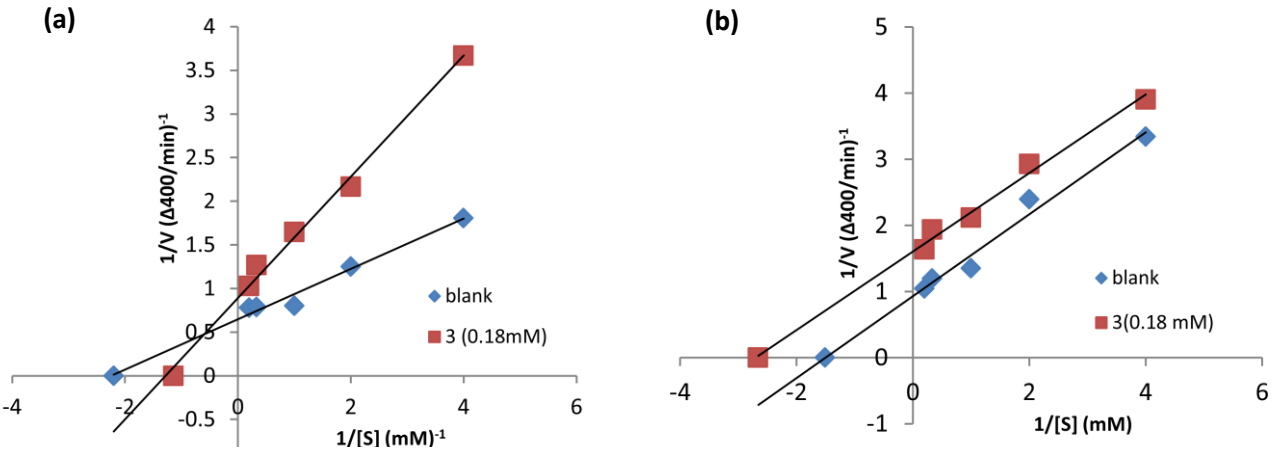

Figure. 5 Lineweaver-Burke plots of compound **3** against yeast (a) and mammalianα-glucosidase (b)

CONCLUSION

Soluchrin (**1**) a secondary metabolite of terrestrial fungi *A. terreus* showed potential inhibitory activity against yeast and mammalian α-glucosidase. The bromination of sulochrin could increase the inhibitory activity against yeast α-glucosidase. These results indicated that sulochrin (**1**) derived from the *A. terreus* terrestrial fungi could be employed as a lead compound for the development of new α-glucosidase inhibitor.

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