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Research Article

Isolation and Identification of *α-Glucosidase* Inhibitor From *Aspergillus terreus* F38

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ABSTRACT

Controlled postprandial glucose level is an important strategy in preventing DM type 2. Inhibitors of α-glucosidase have been postulated to be useful agents in managements of DM type 2. This study aims to isolate and identify of α-glucosidase inhibitor from*Aspergillus terreus* F38 by liquid fermentation. The mycelium extract of *A. terreus* F38showed strong activity against α-glucosidase with IC_{50} value of 9.65µg/mL. Separation and purification of mycelium ectract yielded compound **I** (Butyrolactone III). The structure was establish on the basis of spectral analysis, according to the data obtained by NMR and LCMS-MS experiments. Compound **I** showed potential activity against α-glucosidase with IC_{50} value of 13.87 µg/mL. Therefore, the metabolites from *A. terreus* F38 can be used as lead compound to design potent α-glucosidase inhibitory agents.

Key words: α **Keyword:** Glukosidase inhibitior, *Aspergillus terreus* F38, butyrolactone III

INTRODUCTION

The number of diabetes mellitus patient increases every year all around the world including Indonesia. The rising prevalence of diabetes has become a major problem worldwide, in 2015 it was estimated that there were 415 million people with diabetes aged 20-79 years was predicted to rise to 642 million by 2040. Three quarters (75%) of those with diabetes were living in low- and middle income countries (Ogurtsova, *et al.,* 2017). Non-communicable diseases are estimated to account more than 50% of all deaths in Indonesia. Cardiovascular disease contributed to 30% of the total number of deaths followed by cancers (13%), and diabetes (3%) (Soewondo *et al.,* 2013). The epidemiological and nutritional transitions have played a major role in these trends.

Postprandial hyperglycemia harbors an important role in DM type 2 progresses and specific complication such as diabetic neuropathy, retinopathy, and cardiovascular diseases (Shibano, *et al.,* 2008).Therefore, controlled postprandial glucose level is an important strategy in preventing DM type 2. Therapeutic approach can be done by delaying glucose absorption through inhibiting carbohydrate hydrolyzing enzyme such as α-glucosidase within the digestive organs (Anurakkun, *et al*., 2007). Clinically used drugs nowadays consist of acarbose and miglitol which inhibits glycosidase such as α-glucosidase and α-amylase. However, several hypoglycemic agents have limitations such as side effects and increasing diabetes complication. Main side effects from α-glucosidase inhibitor in gastrointestinal tract are bloating, nausea, and diarrhea. Natural α-glucosidase inhibitor drug that comes from natural sources can be used as therapeutic approach to treat postprandial hyperglycemia for its assumed lower side effect and more affordable price compared to synthetic drugs (Sudha, *et al*., 2011).

Previous our study has shown that fungi of *Aspergillus* sp, can produce an α-glucosidase inhibitors. Butyrolactone I, II, and three synthetic butyrolactone I derivatives were isolated from *A. Terre us* MC751 cultured in Czapek-dox broth, showed potential activity toward α-glucosidase (Dewi, *et al.,* 2014), sulochrin and its brominated derivated also produced by *A. terreus* RCC1 by solid state fermentation exhibition of significant activity

against α-glucosidase (Dewi, *et al*., 2018) and *rubrofusarin* showed potential activity inhibited of mamalian α-glucosidase, which isolated from *A. aculeatus* cultured in potato dextrose broth (Dewi, *et al.,* 2016). Continuing of our efforts to isolate potential a-glucosidase inhibitors from terestrial fungi, the objective of this study was to isolated active compound from *A. terreus* F38 which cultured in potato dextrose broth.

MATERIAL AND METHODS

General Instrument and reagent

UV-Vis absorption spectra of the active compound in methanol were recorded on a Agilent Techn. Carry 60 spectrophotometer. ESI-MS analysis was conducted by LCMS/ MS Xevo G2-XS QTOF (Waters). The nuclear magnetic resonanse (NMR) spectra were recorded at 500MHz for 1H and 125MHz for 13°C on a JEOL JNM-ECA 500 using Acetone-*d6* as solvent, with TMS as internal standard. 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 F254) 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). Potato dextrose broth (PDB), peptone, malt extract, and Czapek-dox broth were purchased from DIFCO.

Microorganisms and culture conditions

Thefungal was obtained from the Indonesian Culture Collection of the Study Center for Biology – LIPI. The strains was prepared on potato dextrose agar plates and stored at 4°C. The isolate was kept by monthly transfer onto PDA (Potato Dextrose Agar) and incubated at 30°C for 7 days in the dark.

Optimization of the culture medium was conducted by culturing the *A. terreus* F38 in three different media: PDB (250 g potatoes boiled in liquid for 1h, filtered and then added with water up to 1 Land 2% glucose.PMP (PDB medium added with 1.0% malt and 0.1% peptone) and Czapek-dox (CDB) (3% sucrose, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05 $MgSO_4.7H_2O$, 0.05% KCl, 0.001% $FeSO_4.7H_2O$) incubated for 10 days in room temperature at static condition. After incubation day was over, mycelium and broth were separated and each was extracted by ethyl acetate (EtOAc), before then dried by rotary evaporator. The culture broth was prepared by 3 rounds of extraction for each broth (500mL) with EtOAc (2x200mL). The dried extract obtained was assessed for α-glucosidase inhibitory activity.

Fermentation, extraction, and isolation

To isolate the active compound, fermentation was carried out to obtain a total volume of 30 L PDB culture broth of *A. terreus* F38. The medium is incubated for 10 days in room temperature at static condition. After incubation, mycelium and broth were separated and each was extracted with 5x1000mL EtOAc. The crude extract was purified by column chromatograph using silica gel G60 (70-230 mesh) using a stepwise gradient *n*-hexane: EtOAc, to EtOAc: MeOH to obtain seven fractions (F1- F7). Fraction 5(F5) further sephadex chromatographed using $CHCl_3$: MeOH (1:1) as system solvent to give fourteen fractions (F5.1-F5.14). Compound **I** (8.9mg) was obtained from purification from sub-fraction F5.8. The pure compounds were identified by instrumental analysis.

Compound **I**: as a yellowish gum. UV spectra (MeOH) λ_{max} 304, LCESI-MS $[M+H]^+$ m/z 441.2843, calcd for $C_{24}H_{24}O_8$]. FTIR (KBr) Vmax 3334, 3086, 2937, 1741, 1093cm^{-1}. (Table III).

Activity test of α-glucosidase inhibitor

α-Glucosidase inhibitory activity was evaluated according to the previously reported method (Dewi *et al.,* 2014). Briefly, 250μL α-Glucosidase, 495 μL of 0.1 M phosphate buffer (pH 7.0), and 5μL of various concentrations of samples in DMSO (50-200μg/mL) were pre-incubated at 37°C for 5min. The reaction was initiated by the addition of 250μL of 5mM *p*-NPG (Wako, Osaka, Japan).

Table I. α-Glucosidase inhibitory activity from three different culture broths of *A. terreus* F38

No	Media	Extract	Extract dried weight (mg/100 mL)	IC_{50} (µg/mL)
1.	PDB	M	34	9.65
		F	17	19.89
2.	PMP	M	55	13.34
		F	19	77.18
3.	CDB	M	26	13.33
		F	11	47.89

Annotation: M = mycelium; F = filtrate

Table II. α-Glucosidase inhibitory activity of Fractions 1-7

No	Fraction	Fraction weight (g)	IC_{50} (µg/mL)
1.	F1	2.905	>1000
2.	F2	0.600	>1000
3.	F3	0.150	426.12
4.	F4	5.8	263.41
5.	F5	1.970	1.48
6.	F6	0.018	202.64
7.	F7	0.800	6.23

The reaction was continued for 15min leter at same condition and stopped by adding 1 mL of 0.1M Na_2CO_3. α-Glucosidase activity was determined by measuring the release of *p*-NPG at 400nm. Individual blanks for test samples were prepared to correct background absorbance where the enzyme was replaced with 250µL of phosphate buffer. % Inhibition = (A - B)/A 9 100, where A was the absorbance of the control reaction and B was the absorbance in the presence of the sample IC_{50} is calculated by using linier regression equation with sample concentration as x axis and inhibition % as y axis.

RESULT AND DISCUSSION

In order to enhanced production of active compound, we initialy evaluated α-glucose inhibitory activity of three differences EtOAC extract of *A. terreus* F38 which cultured in PDB, PMP, and Czapek-dox broth (Table I).

The result showed that mycelium extract and filtrate extract from PDB has the highest activity than others, with IC_{50} value of 9.65 and 19.89µg/ml, respectively. Furthermore, this result showed that intracellular metabolite of *A. terreus* F38 in PDB medium produce highest α-glucosidase inhibitory activity. The addition of nitrogen source (PMP) or ion source (CDB) did not influence on α-glucosidase inhibitory activity compared to PDB medium. Based on that reason, PDB medium is chosen to be used in further study.

On the next step of the study, fermentation was done with 30L scale medium to obtain mycelium extract ±13.0g and 8g of filtrate extract. The separation of mycelium EtOAc extract (± 12.5g) was conducted by vacuum column chromatography with gradual polarity increase to obtain 7 fractions depending on TLC profile. Each fraction obtained was tested against α-glucosidase (Table II).

Based on the test (Table II), the fraction five (F5) and fraction seven (F7) showed strong activity against α-glucosidase with IC_{50} value of 1.48 and 6.23µg/mL, respectively. F5 showed not only the highest inhibitory activity compared to other fractions, but also has relatively high yield (1.97g) which allows further separation and purification. Fraction five (F5) was further purification through column chromatography with silica gel G60, sephadex LH60, and recristalization to obtain in the shape of yellow gum compound **I** (8.9mg).

Table III. Comparison of ^{13}C and ^{1}H data of Butyrolactone I and Butyrolactone III (400MHz, in $CDCl_3$ (Cazar, *et al.,* 2005) to compound **I** (500MHz, in Acetone-*d6*)

	Butyrolactone I*		**Butyrolactone III***		**Compound I****	
	^{13}C	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**
1	169.55	-	168.13	-	169.00	-
2	137.37	-	138.34	-	140.0	-
3	128.51	-	127.75	-	121.0	-
4	86.21	-	84.94	-	86.00	-
5	169.91	-	169.96	-	170.89	-
6	38.67	3.58	38.24	3.53	39.25	3.45
		3.52		3.48		3.45
1’	122.06	-	121.23	-	124.30	-
2’	129.16	7.65	129.02	7.62	130.03	7.61
3’	116.09	7.65	116.07	6.91	116.59	6.97
4’	156.98	-	158.12	-	158.85	-
5’	129.16	6.90	129.00	6.90	130.20	6.97
6’	116.09	7.65	116.07	7.62	116.07	7.61
1”	124.60	-	123.43	-	124.90	-
2”	131.85	6.51	131.35	6.5	130.17	6.45
3”	126.82	-	124.82	-	122.78	-
4”	153.16	-	156.45	-	153.16	-
5”	115.12	6.52	114.83	6.50	116.59	6.47
6”	129.61	6.58	131.35	6.57	130.17	6.53
7”	28.86	3.12	26.63	2.37	26.20	2.54
8”	121.63	5.08	65.44	2.41	69.78	2.76
9”	134.10	-	58.32	-	59.78	-
10”	25.68	1.65	23.31	1.24	20.25	1.14
11”	17.17	1.70	23.31	1.25	20.25	1.28
5-OMe	53.60	3.80	53.52	3.78	53.82	3.79

The ^{1}H and ^{13}C NMR spectra of compound **I** has similar patern with butyrolactone I, its major compound from *A. terreus* (Cazar*, et al.,* 2005 and Dewi, *et al.,* 2014). The ^{1}H NMR spectrum revealed two methyl singlets at δ_H 1.14 and 1.28, presence of methoxy group (-OCH3) at δ_H3.79. Three aromatic proton signal at δ_H 6.53, 6.47, and 6.45, being for 1,2,4-trisubtituted phenol along with two doublets at δ_H 7.61 and 6.98 representing 1,4-disubtituted phenolic moity.

Supported by the signals at δ_C three methine Signals at δ_C 132.4, 128.3, and 115.1 for 1,2,4-trisubtituted benzene ring and 130.2 and 116.7 for 1,4-disubtituted phenolic, respectively. The ^{13}C NMR also showed the presence of two ester carbonyls at δ_C 170.89 and 169.00. The significant difference observed in the NMR spectra between butyrolactone I and compound **I** was appeared high field shift of the C8 and C9 carbon signal at 59.78 and 69. 78 ppm from olefin carbon signals of butyrolactone I at δ_C 132.5 and 123.4ppm. Indicating presence of an oxygeneted carbon (epoxy group). From LCMS-MS, compound **I** has (m/z) 441 $(M+H)^+$ indicates the molecular weight of the compound corresponds to molecular formula $C_{24}H_{24}O_8$, which means has one more oxygent atom than butyrolactone I. This result supported presence of epoxy group.

Based on the NMR data, supported by ESI-MS data and reference (Table III),

compound I was fully consistent with butyrolactone III (Figure 1). Butyrolactone III is reported as the synthetic epoxidation result of butyrolactone I with m-chloroperbenzoic acid (Rao, *et al*., 2000; Cazar, *et al*., 2005). Butyrolactone I is the main compound or metabolite produced by *A. terreus,* while butyrolactone III has never been reported as direct isolation product from *A. terreus* F38 extract. The biological activity of butyrolactone III that has been reported is cytotoxic activity against MCF7 cell (Rao, *et al*., 2000), and *phytotoxic* against *Lactuca sativa* and *Panicum millaceum* with IC_{50} value being $5x10^{-4}$ M and $5x10^{-4}$ M respectively, while the comparison compound *acetochlor* has IC_{50} value of 1 $x10^{-5}$M (Cazar, *et al*., 2005). Activity of this compound as α-glucosidase enzyme inhibitor has never been reported.

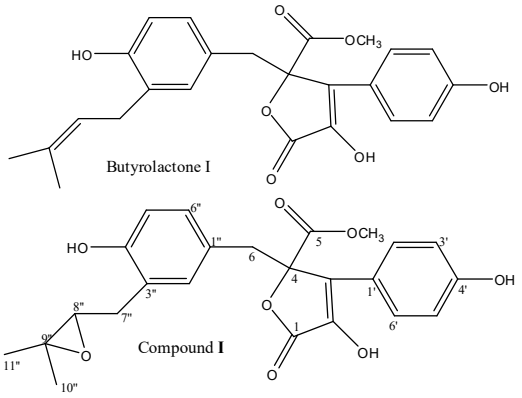

Figure 1. Chemichal structure of butyrolactone I and compound I (butyrolactone III)

CONCLUSION

A. terreus F38 cultured in potato dextrose broth (PDB) ethyl acetate extract showed the highest α-glucosidase inhibitory activity compared to extract obtained from potato malt peptone (PMP) and Czaptek-dox broth (CDB). Butyrolactone III was isolated from *A. terreus* F38 as active compound against α-glucosidase with IC_{50} value of 13,87µg/mL. Therefore, the metabolites from *A. terreus* F38 can be used as lead compound to design potent α-glucosidase inhibitory agents.

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