

Antidiabetic and Antioxidative Activities of Butyrolactone I from *Aspergillus terreus* MC751

Rizna Triana Dewi, Sanro Tachibana, and Ahmad Darmawan

Abstract—The bioassay-guided isolation and purification of an ethyl acetate extract of *Aspergillus terreus* MC751 led to the characterization of butyrolactone I as an antidiabetic and antioxidant. The antidiabetic activity of butyrolactone I was evaluated by α -glucosidase and α -amylase inhibition assays. Butyrolactone I demonstrated significant concentration-dependent, mixed-type inhibitory activity against yeast α -glucosidase with an IC_{50} of 54 μ M. However, the compound exhibited less activity against rat intestinal α -glucosidase and α -amylase. This is the first report on α -glucosidase inhibitory activity of butyrolactone I. The antioxidative activity of butyrolactone I was evaluated based on scavenging effects on 1,1-diphenyl-2-picrylhydrazyl (DPPH) (IC_{50} = 51 μ M) and hydrogen peroxide (IC_{50} = 141 μ M) radicals as well as a reducing power assay. The results suggest that butyrolactone I is a promising antidiabetic as well as antioxidant and should be considered for clinical trials.

Keywords—*Aspergillus terreus* MC751, antidiabetic, antioxidant, Butyrolactone I.

I. INTRODUCTION

HYPERGLYCEMIA, a classical risk factor in the development of diabetes, results in the generation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress in a variety of tissues [1]. The increase in free-radical production and reduction of antioxidative defenses may partially mediate the initiation and progression of diabetes-associated complications [2]. Hence, supplementation with antioxidant can be beneficial for diabetic patients, not only to maintain antioxidant levels in the body but also to treat the long-term complications that can arise [3].

Several management strategies have been proposed for the early stages of dysglycemia with the aim of preventing further development. The inhibition of α -glucosidase and α -amylase, key enzymes in the digestion of carbohydrates, is one way to suppress post-prandial hyperglycemia, by retarding the digestion of oligosaccharides and disaccharides, and delaying glucose absorption as well as reducing glucose levels in

plasma [4]-[5]. Therefore, a combination of α -glucosidase inhibitors and antioxidants would be effective at preventing further development of diabetes mellitus (DM) type 2 [6].

Acarbose, a pseudo-tetrasaccharide isolated from the fermentation broth of *Actinoplanes* spp, is a very popular α -glucosidase inhibitor and has been utilized for the treatment of DM type-2 [7]. Interest in the isolation of α -glucosidase inhibitors from certain microorganisms has increased. For example, validamycin A was isolated from *Streptomyces hygroscopicus* var. *limoneus*, broth of *Bacillus subtilis* B2 also possessed strong α -glucosidase activity [7], the new *N*-containing maltooligosaccharide GIB-638 was isolated from a culture filtrate of *Streptomyces fradiae* PWH638 [8], and Aspergillusol A was isolated from marine-derived fungus *Aspergillus aculeatus* [9]. However, there have been relative few studies on α -glucosidase inhibitors and antioxidants from species of *Aspergillus*.

Previously study, we reported that the ethyl acetate extract of *A. terreus* showed potential inhibitory activity toward α -glucosidase [10] and produced antioxidants [11]. Recently, we found that the ethyl acetate extract of *A. terreus* MC751 showed both α -glucosidase inhibitory and antioxidative effects. Therefore, the objective of this study was to isolate and investigate the antidiabetic and antioxidative compounds from the extract. Antidiabetic activities were investigated using yeast and rat intestinal α -glucosidase, and porcine pancreatic α -amylase. Antioxidative activities were evaluated based on radical scavenging effects on DPPH and hydrogen peroxide radicals as well as reducing power assays.

II. MATERIAL AND METHODS

A. Chemicals

α -Glucosidase [(EC 3.2.1.20)] Type I: from *Sacharomyces cereviceae*, *p*-nitrophenyl α -D-glucopyranoside (*p*-NPG), DMSO, soluble starch, 1,1-diphenyl-2-picrylhydrazyl (DPPH), quercetin dehydrate, potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid, ferric chloride ($FeCl_3$), hydrogen peroxide, and Silica gel (60-200 mesh Wako gel) 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), α -amylase from porcine pancreas (Type VI-B), and 3,5-dinitro salicylic acid, were obtained from Sigma Chemical Co. (St. Louis, MO). All the solvent used in this study were purchased from Wako Pure Chemicals and distilled prior to use.

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B. Methods

1. General experimental procedures

Optical rotation values were measured with a Jasco P-2100 polarimeter. UV-Vis absorption spectra of the active compound in methanol were recorded on a Hitachi U-1600 spectrophotometer. The mass spectra of the compound were measured with high-resolution FAB-MS. The nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for ^1H and 125 MHz for ^{13}C on a JEOL JNM-ECA 500 with TMS as the internal standard. HMQC and HMBC techniques were used to assign correlations between ^1H 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 F₂₅₄ pre-coated plates (Merck 5554) and spots were detected by UV light.

2. Microorganisms and culture conditions

A. terreus MC751 was obtained from the Research Center for Chemistry-Indonesian Institute of Sciences. The stock culture of *A. terreus* was grown on PDA, incubated at 25°C for seven days. Two discs (8mm) of fungal mycelia were used to inoculate 50 mL of Czapek-dox's broth (3% sucrose, 0.2% sodium nitrate, 0.1% K₂HPO₄, 0.05% magnesium sulphate, 0.05% potassium chloride, and 0.001% ferrous sulphate). After 10 day's incubation under static conditions (25°C), the culture broth was filtered through ADVANTEC filter paper no.2 to separate the filtrate and mycelia. The filtrate (16 L) was subjected to shaking with ethyl acetate (EtOAc) at 250 rpm for 20 min 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).

3. Extraction and isolation

The EtOAc extract (1.8 g) was chromatographed on silica gel (35 x 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 eight fractions (F1-F8) based on TLC. α -Glucosidase inhibitor and DPPH free radical-scavenging assays showed that fraction 6 (900 mg) was the most active with an IC₅₀ of 30.01 and 45.27 $\mu\text{g/mL}$, respectively. Fraction 6 was further separated by repeated silica gel column chromatography using stepwise elution with hexane: EtOAc and preparative layer chromatography (PLC) with CHCl₃: Acetone (3:1) to afford butyrolactone I (200 mg), as a yellowish sticky solid. UV spectra (MeOH) λ_{max} 307 (log ϵ 4.3). $[\alpha]_{\text{D}}^{22.5}$ +68.333 (c, 0.3 in MeOH). HRFABMS: $[\text{M}+\text{H}]^+$ m/z 425.1607, calcd for C₂₄H₂₅O₇] 13 degrees of unsaturation. ^1H NMR (Acetone-*d*₆) δ 1.56 (3H, s), 1.64 (3H, s), 3.10 (2H, d, J=6.8), 3.43 (2H, d, J=14.8), 3.76 (3H, s), 5.51 (1H, br, J=7.3), 6.49 (1H, dd, J=8.0), 6.52 (1H, d, J=8.0), 6.53 (1H, d, J=3.4), 6.95 (2H, d, J=9.0), 7.61 (2H, d, J=9.0). ^{13}C NMR (Acetone-*d*₆) δ 170.97 (C-5), 168.72 (C-1), 158.93 (C-4'), 154.81 (C-4''), 139.12 (C-2), 132.48 (C-9''), 132.36 (C-2''), 130.20 (C-6' and C-3'), 129.60 (C-6''), 122.85 (C-1'), 128.27 (C-3''), 124.94 (C-1''), 123.43 (C-8''), 127.95 (C-3), 116.67 (C-5' and C-2'), 115.05 (C-5''), 86.02 (C-4), 53.76 (OCH₃), 39.32 (C-6), 28.62 (C-7''), 25.95 (C-

10''), 17.82 (C-11''). The physical and spectral (one and two dimensional NMR, and FABMS) characteristics of the isolated compound coincided with those of butyrolactone I (α -oxo- β -(*p*-hydroxyphenyl)- γ -(*p*-hydroxy-*m*-3,3-dimethylallylbenzyl)- γ -methoxycarbonyl- γ -butyrolactone I) reported in the literatures [12]-[15].

4. Antidiabetic assay

i. Yeast α -glucosidase inhibition assay

The inhibitory activity for α -glucosidase was assessed as reported by Kim *et al.* [16] with minor modifications. The reaction mixture contained 250 μL of 3mM *p*-NPG and 495 μL of 100mM phosphate buffer (pH 7.0) added to a tube containing 5 μL of sample dissolved in DMSO at various concentrations (5 to 100 $\mu\text{g/mL}$). The reaction mixture was pre-incubated for 5 min at 37°C, the reaction was started by adding 250 μL of α -glucosidase (0.065units/mL) and the incubation was continued for 15 min. The reaction was stopped by adding 1mL of 0.2 M Na₂CO₃. The inhibitory effect on α -glucosidase activity was determined by measuring the amount of *p*-nitrophenol released at λ 400 nm. Individual blanks for test samples were prepared to correct background absorbance where the enzyme was replaced with 250 μL of phosphate buffer. All the tests were run in triplicate.

ii. Rat intestinal α -glucosidase inhibition assay

The inhibitory activity toward rat intestinal α -glucosidase was assayed as described by of Sancheti *et al.* [17] with a slight modification: 0.5 grams of rat-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 s 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 $\mu\text{g/mL}$) 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 rat intestinal α -glucosidase solution was added. The reaction was then terminated by the addition of 1mL of 0.2 M Na₂CO₃. Absorbance readings were 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.

iii. Porcine pancreatic α -amylase inhibition assay

The assay was conducted according to Gowri, *et al.* [18] In brief, 40 μL of test sample (50-200 $\mu\text{g/mL}$) and 160 μL of 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride were incubated with 200 μL of porcine pancreatic α -amylase (0.5 mg/mL prepared in ice-cold distilled water) for 5 min. The reaction was started by adding of 400 μL of a soluble potato starch solution (0.5% w/v in 20 mM buffer). Exactly 3 min later, 400 μL of DNS color reagent was added. The reaction mixture was then incubated in a water bath (85-90°C) for 10 min to develop the color, and cooled to room temperature. Next, 500 μL of reaction mixture was diluted with 1750 μL of distilled water, and absorbance was

measured at λ 540 nm. Individual blanks were prepared to correct background absorbance due to test samples. All the tests were run in triplicate.

The percent inhibition of α -glucosidase and α -amylase was assessed using the following formula: % Inhibition = $[1 - (As/A0)] \times 100$, where A0 was the absorbance of the control reaction and As was the absorbance in the presence of the sample. The IC₅₀ values were calculated from the mean inhibitory values by applying logarithmic a regression analysis.

iv. Determination of the inhibition pattern on α -glucosidase

To evaluate the type of inhibition against yeast α -glucosidase, increasingly higher concentrations of *p*-NPG were used as a substrate in the absence and presence of butyrolactone I. The inhibition type was determined from Lineweaver-Burke plots.

5. Antioxidant assays

i. DPPH radical-scavenging activity

The free radical-scavenging activities of samples were measured by using DPPH according to Yen and Chen [19]. Each sample in methanol (4 mL) with a concentration of 10-200 μ g/mL was mixed with 1 mL of 1 mM DPPH solution in methanol. The mixture was shaken vigorously and left to stand for 30 min in the dark, and then the absorbance was measured at λ 517 nm against a blank. The ability to scavenge the DPPH radical was calculated using the following equation: DPPH-scavenging effect (%) = $[1 - (As/A0) \times 100]$, where A0 was the absorbance of the control reaction and As was the absorbance in the presence of the sample. The percentage of scavenging activity was subsequently plotted against the sample concentration. The half maximal inhibitory concentration (IC₅₀) was calculated from the graph of percent antioxidative activity against sample concentration. The assays were carried out in triplicate and the results expressed as mean values \pm standard deviations. Quercetin was used as a reference compound.

ii. Hydrogen peroxide radical- scavenging activity

A solution (40 mM) of hydrogen peroxide was prepared in phosphate-buffered saline (PBS, pH 7.4). the concentration of hydrogen peroxide was determined spectrophotometrically from absorption at 230 nm with a molar absorptivity of 81 M⁻¹cm⁻¹ according to the method of Yen and Chen [19]. Samples (10-200 μ g/mL) in 4 mL of methanol were added to the hydrogen peroxide solution (0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined 10 min later against a blank solution containing samples in PBS without hydrogen peroxide. The percent inhibitory activity was calculated as follows: hydrogen peroxide-scavenging effect (%) = $[1 - (As/A0) \times 100]$, where A0 was the absorbance of the control reaction and As was the absorbance in the presence of the sample. Quercetin was used as positive control.

iii. Reducing power assay

The reducing power of the extract and pure compound was determined according to the method of Yen & Chen [19]. Various concentrations of samples (10-200 μ g/mL) in 0.5 mL of methanol were mixed with 2.5 mL of 0.2M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [K₃Fe(CN)₆], then incubated at 50°C for 20 min. After 2.5 mL of 10% of trichloroacetic acid was added, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and absorbance was measured at 700 nm. Increasing absorbance of the reaction indicated increasing reducing power. The assay was carried out in triplicate and the results were expressed as mean values \pm SD. Quercetin was used as a standard.

III. RESULT AND DISCUSSION

A. Isolation and characterization of the active compound

The interest in finding effective and safe α -glucosidase inhibitors possessing antioxidative effects from natural sources has increased in order to develop lead compounds for antidiabetic agents. An ideal anti-diabetic compound should possess both hypoglycemic and antioxidative properties [6]. The EtOAc extract of MC751 cultured in Czapek-dox medium showed potential inhibitory activity toward α -glucosidase and antioxidative activity with an IC₅₀ of 37.01 μ g/mL and 45.34 μ g/mL, respectively. The extract was purified further using column chromatography on silica gel to isolate butyrolactone I as the major compound in Fig. 1.

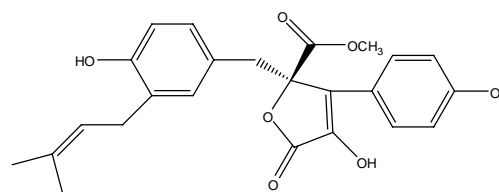


Fig.1 Chemical structure of the isolated compound: α -oxo- β -(*p*-hydroxyphenyl)- γ -(*p*-hydroxy-*m*-3,3-dimethylallylbenzyl)- γ -methoxycarbonyl- γ -butyrolactone (I)

Butyrolactone I seems to be a common metabolite of *A. terreus* and was first isolated in 1977 from *A. terreus* var Africans IFO 8355 [14]. This compound displays several interesting biological activities, such as antitumor effects (cytotoxicity), allergenic effects, inhibition of microbial and plant growth, and both convulsant and anticonvulsant activity [13]. Butyrolactone I was also reported to inhibit an eukaryotic cyclin-dependent kinase (CDK), preventing apoptosis [15]. It also showed inhibitory activities against soybean lipoxigenase and had DPPH radical-scavenging activity [20]. There is no report available on the antidiabetic activities of butyrolactone I, so this study explored their effect in detail and also examined antioxidative activities.

B. Antidiabetic assay

In the present study, the antidiabetic activity of the extract and butyrolactone I was evaluated using α -glucosidase from two different sources (yeast and rat intestinal) and an α -amylase inhibition assay. For general screening of α -glucosidase inhibitors, a yeast enzyme from *S. cereviceae* categorized as a type I α -glucosidase [18] was selected. The extract of *A. terreus* and butyrolactone I showed strong α -glucosidase inhibitory activity with an IC_{50} of 37.01 and 22.86 μ g/mL (or 54 μ M), respectively. However, quercetin showed the strongest inhibition with an IC_{50} of 14.6 μ M in Table I. Butyrolactone I had a significantly greater effect than the extract, therefore it can be assumed that it is the compound responsible for the activity in the extract of *A. terreus* MC751. However, the extract, butyrolactone I, and quercetin all displayed significantly less of an effect on the intestinal α -glucosidase than yeast α -glucosidase as in Fig. 2. It has reported that most yeast inhibitors of α -glucosidase do not show any activity against mammalian α -glucosidases due to differences in the molecular recognition of the target-binding site of the enzyme [21]. The presence of a phenolic hydroxyl group was assumed to contribute to the inhibitory effect of butyrolactone I [4], [22]-[24] moreover, the presence of a lactone moiety also played a role, due to the activity of γ -lactone to inhibit fatty acid synthase and reduce adipose tissue [25].

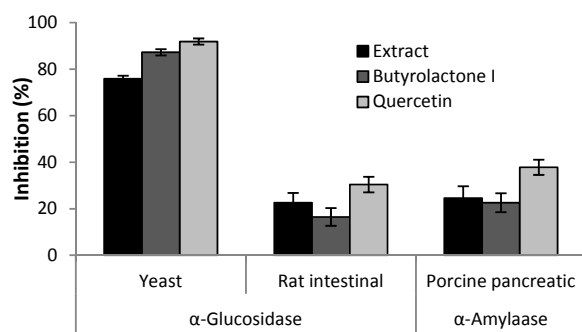


Fig. 2 Inhibitory activities of the extract, butyrolactone I, and quercetin against α -glucosidase and α -amylase activity at a concentration of 100 μ g/mL ($n=3$, mean \pm Sd)

The pattern of butyrolactone's effect on the yeast α -glucosidase, was estimated using Lineweaver-Burke plots and consistent with mixed-type inhibition in Fig. 3. The K_i (inhibition constant) value of butyrolactone I was 55 μ M, determined from Dixon plots. The mixed-type inhibition was characterized by a combination of competitive and non competitive inhibition [22] which indicated that butyrolactone I binds to a site other than the active site of the enzyme and combines with either free enzyme or the enzyme substrate complex, possibly interfering with the actions of both [3]. The inhibitory mechanism of butyrolactone I is similar to that of quercetin [5], [23].

In the porcine pancreatic α -amylase assay, both the samples (extract and butyrolactone I) and quercetin as a

reference exhibited low inhibitory activity in Fig. 2. Pancreatic α -amylase is a key enzyme in the digestive system, catalyzing the initial step in the hydrolysis of starch to a mixture of smaller oligosaccharides, which are further degraded by α -glucosidases to glucose which on absorption enters the blood-stream [18]. Natural inhibitors, which have been shown to inhibit α -amylase activity weakly and α -glucosidase activity strongly, may offer a better therapeutic option for controlling postprandial hyperglycemia with minimal side effects [18], [21]. Therefore, the inhibition of α -glucosidase appears to be a more effective way to control the release of glucose from disaccharides in the gut than α -amylase [18].

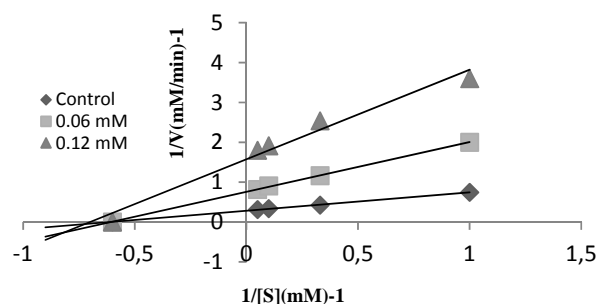


Fig. 3 Lineweaver-Burk plot of yeast α -glucosidase and *p*-NPG in the presence of butyrolactone I at different concentrations of *p*-NPG ($n=3$, mean \pm Sd)

C. Antioxidant assay

There is much evidence that hyperglycemia results in the generation of ROS, ultimately leading to increased oxidative stress in a variety of tissues. Oxidative stress plays an important role in the pathogenesis of late diabetic complications [26]. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals (O_2^-) and hydroxyl radicals (OH^\cdot), as well as non-free radicals (H_2O_2) and singlet oxygen [27]. One of the mechanisms involved in antioxidative activity is the ability to donate a hydrogen atom to a radical, and the propensity for donating hydrogen is a critical factor in free radical scavenging [28]. It is essential to use more than one method to evaluate the antioxidative activity of extracts and isolate compounds.

Free radical-scavenging is a major property of antioxidants [29]. Therefore, we assessed the scavenging effect of the extract and butyrolactone I by using the DPPH radical. DPPH is a stable free radical commonly used to assess the scavenging activity of antioxidants compound [28]. The scavenging activity of butyrolactone I was similar to that of quercetin: the IC_{50} was 21.68 and 11.97 μ g/mL respectively in Fig. 4. Compared to the extract, butyrolactone I had significantly greater scavenging effect on the DPPH radical, and the IC_{50} being consistent with that reported by Sugiyama *et al* [20]. The scavenging mechanism of butyrolactone I was assumed to be the donation of two hydrogen radicals to two molecules of DPPH which produce two molecules of

hydrazine DPPH, followed by phenolic conversion into quinone methide, as occurred in Aspernolid A [20].

Hydrogen peroxide is a relatively unstable metabolic product responsible for the generation of hydroxyl radicals and singlet oxygen, which is formed by the Fenton reaction and initiates lipid peroxidation and causes DNA damage [27],

[29]. Therefore, reducing hydrogen peroxide is important for antioxidative defense in the cell [29]. The extract and butyrolactone I were both capable of scavenging hydrogen peroxide, acting in a manner dependent on concentration in Fig. 4.

TABLE I
ANTIDIABETIC AND ANTIOXIDATIVE ACTIVITIES OF THE EXTRACT AND BUTYROLACTONE I COMPARED TO QUERCETIN AS A REFERENCE

Sample	Antidiabetic (IC ₅₀) ^a			Antioxidant (IC ₅₀) ^a	
	Yeast α -glucosidase	Rat intestinal α -glucosidase	Porcine pancreatic α -amylase	DPPH	H ₂ O ₂
Extract	37.01 μ g/mL	313.36 μ g/mL	257.57 μ g/mL	45.34 μ g/mL	70.66 μ g/mL
Butyrolactone I	54 μ M	> 500 μ M	> 500 μ M	51.13 μ M	141.39 μ M
Quercetin	14.6 μ M	> 500 μ M	460 μ M	39.63 μ M	123.81 μ M

^a Inhibitory activity was expressed as the mean \pm SD of three determinations, and was obtained by interpolation of the concentration inhibition curve.

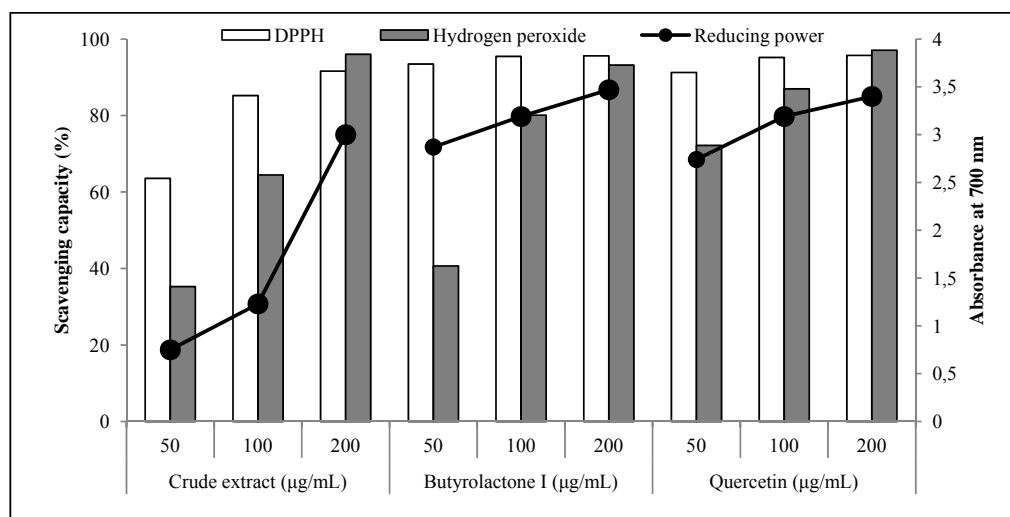


Fig. 4 Antioxidative activity of the extract and butyrolactone I compared to quercetin as a reference ($n=3$, mean \pm Sd)

Reducing power is associated with and may serve as a significant reflection of antioxidative activity. Compounds with reducing power are electron donors, which can act as primary and secondary antioxidants [19]. The reducing power of butyrolactone I increased with the amount of sample (OD value: 0.7-3), similar to quercetin in Fig. 4. Moreover, butyrolactone I was more active than the extract. Some studies reported that the activity of antioxidants corresponded to the number of hydrogens available for donation by hydroxyl groups [29] the radical-scavenging effect of butyrolactone I might be due to the hydroxyl group substituent.

IV. CONCLUSION

In conclusion, the present study revealed that butyrolactone I, a major compound in the ethyl acetate extract of *A.terreus*, was a potential antidiabetic exhibiting inhibitory activity against α -glucosidase with an IC₅₀ of 22.86 μ g/mL (54 μ M)

and a pattern of mixed inhibition. Furthermore, this compound exhibited different levels of antioxidative activity in assays for scavenging the free radical DPPH, scavenging hydrogen peroxide, and reducing power. The results suggest that butyrolactone I is a promising antidiabetic as well as antioxidant and should be considered for clinical studies.

ACKNOWLEDGMENT

The authors are grateful to Dr. M. Hanafi from the Research Center for Chemistry-Indonesian Institute of Sciences, to provide the *A. terreus* culture for this study. We thank Dr. LBS. Kardono for general discussion about this study.

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