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学位論文全文に代わる要約
Dissertation Abstract

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学位論文題目：
Title of Dissertation

Isolation and Characterization of α-Glucosidase Inhibitor and Antioxidant Compounds from *Aspergillus terreus*
（*Aspergillus terreus* からの α-グルコシダーゼ阻害物質および抗酸化物質の単離と特性評価）

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Diabetes mellitus (DM) is one of the most common chronic diseases is known to be affecting people of both developed and developing countries and continues to increase in numbers and significance, as changing lifestyles lead to reduced physical activity, and increased obesity. There were approximately 285 million adult aged 20-79 years with diagnosed DM in 2010, and will increase to 439 million adults by 2030. Between 2010 and 2030, there will be 69% increase in number of adults with diabetes in developing countries and a 20% increase in developed countries. Diabetes mellitus can classify into several types, including type 1 and type 2. Type 1 diabetes, which accounts for only 5–10% of those with diabetes, particularly children and adolescents, may present with ketoacidosis as the first manifestation of the diseases. Type 2 DM is reported to make up 90-95% of all diabetic cases, which characterized by insulin resistance, relative insulin deficiency, and hyperglycemia.

There is considerable evidence that hyperglycemia results from the generation of reactive oxygen species (ROS) production, ultimately leading to increased oxidative stress in a variety of tissues such as nepropathy, retinopathy, neuropathy, micro- and macro-vascular damage. Several anti-hyperglicemic agents are available for the treatment of type 2 DM, including intestinal absorption inhibitors, biguanides, insulin secretagogues, thiazolidindediones (TZDs), and insulin itself. Intestinal absorption inhibitors, such as α-glucosidase inhibitors and lipase inhibitors, play a relatively minor role in the treatment of type 2 DM but have a significant role in the prevention of this disease in high-risk populations. Therefore, the ideal antidiabetic compound should possess both hypoglycemic and antioxidant properties.

The search for α-glucosidase inhibitor from nature led to the discovery of acarbose, a pseudo tetrasacharide isolated from the fermentation broth of Actinoplanes spp. SE-50, has been utilized as medicine for treatment of type 2 diabetes mellitus. Interest in the isolation of α-glucosidase inhibitors from certain

microorganisms has increased due to fast growing characteristic of microorganisms. Therefore, microorganisms are an important source for screening α-glucosidase inhibitors.

Aspergillus terreus is ubiquitous fungus isolated from both marine and terrestrial environments, however common in tropical or sub–tropical areas. The compounds isolated from *A. terreus* mostly posses' pharmacological and commercial values, such as lovastatin is one of the antihyperlipidemic drugs, which inhibits the cholesterol biosynthesis and is a major drug agent in the treatment of heart disease and atherosclerosis. However, there have been relative few studies on α-glucosidase inhibitors and antioxidants from *A. terreus.*

Therefore the aims of the study are to isolation and characterization the active compounds from four selected isolates of *A. terreus* guided by inhibitory on yeast α-glucosidase and antioxidant assays. In study from selection medium and growth condition revealed that each isolates of *A.terreus* showed potential inhibitory on α-glucosidase and antioxidant activities depend on culture medium and growth conditions. *A. terreus* LS01 showed potential antioxidant activity when cultured on potato malt peptone (PMP) media under shaking condition for seven days, while *A. terreus* RCC1 showed potential activity of both inhibitory on α-glucosidase and antioxidant activities when cultured in PMP medium on shaking for fifteen days. *A. terreus* MC751 and LS07 showed both the potential activities of α-glucosidase inhibitory and antioxidant when cultured in Czapek-dox (Cz) media at static condition for ten days and fifteen days, respectively.

The fungus *A. terreus* LS01 was isolated from the Teluk Kodek, Pemenang area, West Nusa Tenggara Province, Indonesia. It was identified as *Aspergillus terreus* Thom., according to its morphological characteristics and 28S rDNA sequence. The fungus has been deposited in the Microbial Collection (LIPIMC) Microbiology Division Research Center for Biology, Indonesian Institute of Sciences. It was prepared on potato dextrose agar plates and stored at 4^{o}C. The liquid culture experiments were conducted in a 250 mL Erlenmeyer-flask containing 50 mL of PMP medium (2.4% potato dextrose broth, 1% malt extract, and 0.1% peptone). The flasks were incubated at 25^{o}C with shaking at 100 rpm for seven days. EtOAc (3x1 L) was added into the culture broth (3 L) and extracted for 20 min by vigorously shaking.

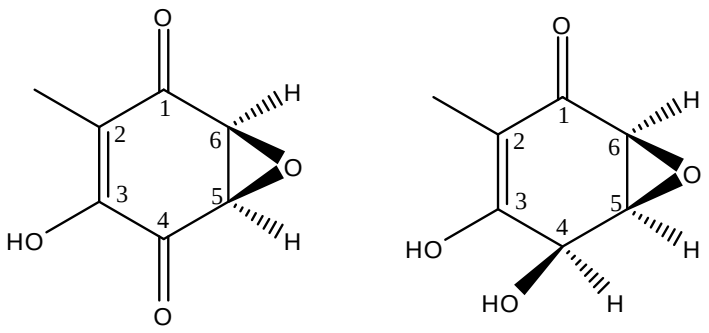
The EtOAc extract (800 mg) obtained from the liquid fermentation of *A. terrus* LS01 was subjected to column chromatography on silica gel 60 (column length 25 cm; internal diameter 3 cm) using a stepwise gradient from *n*-hexane: EtOAc 95:5 v/v (Fraction1, 58 mg); *n*-hexane: EtOAc 85:15 v/v (Fraction 2, 90 mg); *n*-hexane: EtOAc 75:25 v/v (Fraction 3, 250 mg); *n*-hexane: EtOAc 1:1 v/v to 100% EtOAc (Fraction 4, 140 mg) and EtOAc: MeOH 95:5 – 90:10 v/v (Fraction 5, 180 mg). The obtained fraction which showed the highest antioxidant activity (F-3) was further purified with column chromatography on silica gel 60 with a stepwise

gradient from *n*-hexane: EtOAc 9:1 (Fraction 3.1, 5 mg); *n*-hexane: EtOAc 8:2 – 7:3 (Fraction 3.2, 170 mg); *n*-hexane: EtOAc 1:1 (Fraction 3.3, 65 mg). Fraction F3.2 was recrystallized from EtOAc to yield pale yellow needles (compound **1,** 77 mg). Fraction F3.3 was recrystallized from acetone to yield colorless solid (compound **2,** 30 mg). The pure compounds were identified by instrumental analysis.

*Compound **1.*** (1S,6R)-4-hydroxy-3-methyl-7-oxabicyclo[4.1.0]hept-3-ene-2,5-dione *(Terreic acid).* Pale yellow needles, m.p. 126-127^{o}C (lit. m.p 127-127.5^{o}C (Sheehan *et al*., 1958)). UV spectra (MeOH) λ_{max} (log ε) 213 (4.03) and 314 (3.88). $[\alpha]_D^{28.6}$-34 (c, 0.046 in MeOH). ^{1}H (500 MHz; $CDCl_3$, δ-values) δ: 1.93 (s, 3H); 3.86 (d,1H, J=3.9); 3.89 (d,1H, J=3.35); 6.87 (s,1H) and ^{13}C NMR (125 MHz; $CDCl_3$, δ-values): 8.9, 51.6, 53.8, 120.5, 151.9, 187.55, 190.8. HRFABMS: $[M+H]^+$ m/z 155.0358 (cald. for $C_7H_7O_4$, 157.0342).

*Compound **2.*** (1S, 2S, 6S)-2,5-dihydroxy-4-methyl-7-oxabicyclo[4.1.0]hept-4-en-3-one [(±)-*terremutin*]. Colorless solid, m.p 164-166^{o}C (lit. m.p 163-165^{o}C (Read & Ruiz, 1970)). UV spectra (MeOH) λ_{max} (log ε) 272 (4.05). $[\alpha]_D^{28.6}$-283 (c, 0.16 in MeOH). ^{1}H (500 MHz; Acetone-d_6) δ: 1.65 (s, 3H); 3.34 (d, 1H, *J*=2.55); 3.64 (dd,1H, *J*=1.3); 4.59. ^{13}C NMR (125 MHz; Acetone-d_6) δ: 7.5, 52.4, 55.3, 66.2, 108.9, 168, 2. HRFABMS: $[M+H]^+$ m/z 157.0498 (cald. for $C_7H_9O_4$, 157.0498).

Based on UV-Vis, HRFABMS, ^{1}H and ^{13}C NMR data, **1** was identified as (1S,6R)-4-hydroxy-3-methyl-7-oxabicyclo[4.1.0]hept-3-ene-2,5-dione *(Terreic acid)* (Figure 1). The ^{1}H and ^{13}C NMR spectra of compound **1** and **2** were similar. Based on the UV spectra, MS and ^{1}H and ^{13}C NMR data, compounds **2** was identified as (1S, 2S, 6S)-2,5-dihydroxy-4-methyl-7-oxabicyclo[4.1.0]hept-4-en-3-one [(±)-*terremutin*] (Figure 1). Terreic acid (**1**) and terremutin (**2**) exhibited the highest level of DPPH free radical scavenging activity with IC_{50} values of 115.0 and 114.0 μM, respectively. This is the first report on antioxidative activity of terreic acid (**1**) and terremutin (**2**) from *A. terreus.*



Terreic acid (**1**) Terremutin (**2**)

Figure 1. Chemical structure of the antioxidants isolated from *Aspergillus terreus* LS01

The fungus *A. terreus* MC751 was isolated from a leaf litter in Lawu Mountain, Central Java province, Indonesia, in July 2006. This fungus was identified as *A. terreus* on the basis of the sequence data of ITS rDNA. This fungus was deposited in the Microbial Culture Collection (LIPI-MC), Research Center for Biology, Indonesian Institute of Sciences. The bioassay-guided isolation and purification of an EtOAc extract of *A. terreus* MC751 led to the isolation of compound **3** as an antidiabetic and antioxidant.

Compound **3**, as a yellowish gum. UV spectra (MeOH) λ_{max} 307 (log ε 4.3). $[\alpha]_D^{22.5}$+68.333 (c, 0.3 in MeOH). HRFABMS: $[M+H]^+$ m/z 425.1607, calcd for $C_{24}H_{25}O_7$] 13 degrees of unsaturation. ^{1}H (Acetone-*d6*) 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-*d6*) δ 171.0 (C-5), 168.7 (C-1), 158.9 (C-4'), 154.8 (C-4''), 139.1 (C-2), 132.5 (C-9''), 132.4 (C-2''), 130.2 (C-6' and C-3'), 129.6 (C-6''), 122.9 (C-1'), 128.3 (C-3''), 124.9 (C-1''), 123.4 (C-8''), 128.0 (C-3), 116.7 (C-5' and C-2'), 115.1 (C-5''), 86.0 (C-4), 53.8 (OCH_3), 39.3 (C-6), 28.6 (C-7''), 26.0 (C-10''), 17.8 (C-11'').

Structure of compound **3** was further deduced on the basis of HMBC experimental data and comparison with literature, which confirms that compound **3**, coincided with butyrolactone I (α-oxo-β-(p-hydroxyphenyl)-γ-(p-hydroxy-m-3.3-dimethylallylbenzy l)-γ-methoxycarbonyl-γ-butyrlactone I) (Figure 2).

The compound **3** demonstrated significant concentration-dependent, mixed-type inhibitory activity against yeast α-glucosidase with an IC_{50} of 52.2 μM.. The antioxidative activity of compound **3** evaluated based on the scavenging effects on DPPH with IC_{50} value of 51.4 μM. This is the first report on α-glucosidase inhibitory activity of butyrolactone I (**3**). Further studies revealed that the production of compound **3** and activities of EtOAc extract of MC751 increased when Czapek-dox medium was added with 0.5% yeast extract (CzY) and incubated under static condition for 15 days. The EtOAc extract was successively subjected to silica gel column chromatography to afford compounds **3** and **4** as major constituents.

Compound **4**: Colorless gum, UV (MeOH) λ_{max} nm (log ε): 307.5 (4.17). $[\alpha]_D^{28.7}$ +4.78 (c=0.45, Acetone). ^{1}H NMR (Acetone-*d6*) δ 3.40 (2H, d, J=14.8, H-6), 3.76 (3H, s, 5-OCH_3), 6.59 (2H, d, J=8.0, H-3'',5''), 6.69 (2H, d, J=8.0, H-2',6'), 6.98 (2H, d, J=8, H-3',5'), 7.66 (2H, d, J=8.0, H-2'',6''). ^{13}C NMR (Acetone-*d6*) δ 170.9 (C-5), 168.8 (C-1), 158.9 (C-4'), 157.4 (C-4''), 139.3 (C-2), 132.3 (C-2'and C-6') 130.1 (C-2'' and C-6''), 128.0 (C-1''), 124.9 (C-1'), 122.8 (C-3), 115.5 (C-3' and C-5'), 116.7 (C-3'' and C-5''), 85.97 (C-4), 53.8 (OCH_3), 39.2 (C-6). FABMS: $[M+H]^+$ m/z 357 for $C_{19}H_{17}O_7$.

Compound **4** was isolated as colorless gum and its molecular formula was assigned to be $C_{19}H_{16}O_7$ based on FAB-MS analysis (m/z 356), indicating 12 degrees of unsaturation. The ^{1}H NMR spectrum data of **4** showed four o-doublet (J=8 Hz) each of 2 protons at $_\delta$H 6.59, 6.69, 6.98, and 7.66, being for 1-4-disubstituted aromatic residue. A methoxy group at $_\delta$H 3.76, and doublet of an AB methylen group at $_\delta$H 3.40, this spectra resemble those of butyrolactone I (**3**). However, no signal of prenyl group was apparent in the ^{1}H NMR spectrum. Analysis of the ^{13}C NMR spectrum and the information from 2D NMR data (HMQC and HMBC) were coincided with butyrolactone II [methyl-4-hydroxy-2-(4-hydroxybenzyl)-3-(4-hydroxyphenyl)-5-oxo-2,5-dihydrofuran-2-carboxylate] (Figure 2).

In order to verify this structure and to study the structure activity relationship (SAR), compound **3** was derivatized by cyclization and acetylation.

Compound **3a**: Yellowish solid crystal, mp: 93-95^{o}C, UV (MeOH) λ_{max} nm (log ε): 307.5 (4.92); 226.5 (4.85); $[\alpha]_D^{28.7}$ +88.73 (c=0.58, $CHCl_3$). ^{1}H NMR (Acetone-*d6*) δ 1.16 (6H, s, H10”,11”), 1.63 (2H, br, J=6.5, H8”), 2.50 (2H, m, H7”), 3.36 (2H, J=15, H6), 3.68 (3H, s, 5-OMe), 6.38 (1H, s, H2”), 6.50 (1H, s, H5”), 6.56 (1H, d, J=3.4, H6”), 6.86 (2H, d, J=9.0, H3’, 5’) , 7.65 (2H, d, J=9.0, H2’, 6’). ^{13}C NMR (Acetone-*d6*) δ 173.5 (C-5), 172.5 (C-1), 157.9 (C-4’), 154.3(C-4”), 144.9 (C-2), 75.0 (C-9”), 126.4 (C-2”), 129.6 (C-6’ and C-2’), 125.5 (C-6”), 133.0 (C-1’), 117.5 (C-3”), 130.6 (C-1”), 33.8 (C-8”), 121.2 (C-3), 116.6 (C-5’ and C-3’), 123.7 (C-5”), 86.8 (C-4), 53.8 (OCH_3), 40.0 (C-6), 23.3 (C-7”), 27.4 (C-10”), 27.6 (C-11”). FABMS: $[M+H]^+$ m/z 425 for $C_{24}H_{25}O_7$.

Compound **3b**: Colorless gum: UV ($CHCl_3$) λ_{max} nm (log ε): 281 (3.74). $[\alpha]_D^{20}$ +7.154 (c=1.3, $CHCl_3$). FABMS: $[M+H]^+$ m/z 551 for $C_{30}H_{31}O_{10}$.

Compound **3c**: Colorless gum, UV ($CHCl_3$) λ_{max} nm (log ε):294 (3.84). $[\alpha]_D^{20}$ +39.957 (c=1.15, $CHCl_3$). FABMS: $[M+H]^+$ m/z 509, for $C_{28}H_{29}O_9$.

Conversion of compound **3** to **3a** was observed as indicated by TLC ($CHCl_3$/MeOH, 9:1). The molecular formula of compound **3a** was found to be identical with that of **3** based on FABMS ($C_{24}H_{25}O_7$). The ^{1}H NMR spectrum showed a 4H A_2B_2 system at δ_H 6.86 (2H, *J*=9.0) and 7.65 (2H, *J*=9.0) indicating the presence of an *para*-disubstituted phenyl group and three aromatic proton signal of a 1,2,4-trisubstituted phenol at δ_H 6.56, 6.50, and 6.38 in **3a**. One methoxy singlet at δ_H 3.68 and two methyl siglet at δ_H 1.160 and 1.63. The ^{1}H and ^{13}C NMR of compound **3a** was similar with **3**. The significant difference observed in the NMR spectra between compound **3a** and **3** was the absence of an olefininc proton signal, indicating the compound **3** was converted to **3a**.The MS and NMR spectral data of compound **3a** were fully consistent with those for Aspernolide A [methyl 2-((2,2-dimethylchroman-6-yl)methyl)-4-hydroxy-3-(4-hydroxyphenyl)-5-oxo-2,5-dihydrofuran-2-carboxylate].

Compound **3** was treated with acetic anhydride in pyridine to give butyrolactone I 2,4',4''-triacetate (**3b**) [methyl-4-acetoxy-2-(4-acetoxy-3-(3-methylbut-2-enyl)benzyl)-3-(4-acetoxyphenyl)-5-oxo-2,5-dihydrofuran-2-carboxylate] and butyrolactone I 4',4''-diacetate (**3c**) [methyl 2- (4-acetoxy-3-(3-methylbut-2-enyl)benzyl) -3-(4-acetoxyphenyl)-4-hydroxy-5-oxo-2,5-dihydrofuran-2-carboxylate]. The structures of compound **3b** and **3c** were determined from MS and NMR (1 and 2D) data. A comparison of the ^{1}H NMR spectra of compounds **3**, **3b**, and **3c** showed a similar pattern.

All isolated and derivatives compounds from *A. terreus* MC751 in CzY are shown in Figure 2. The compounds were examined for α-glucosidase inhibitory and antioxidant activities. In the present study, the antidiabetic activity of the extract and butyrolactone I was evaluated using α-glucosidase from yeast (*Saccharomyces ceriviseae*).

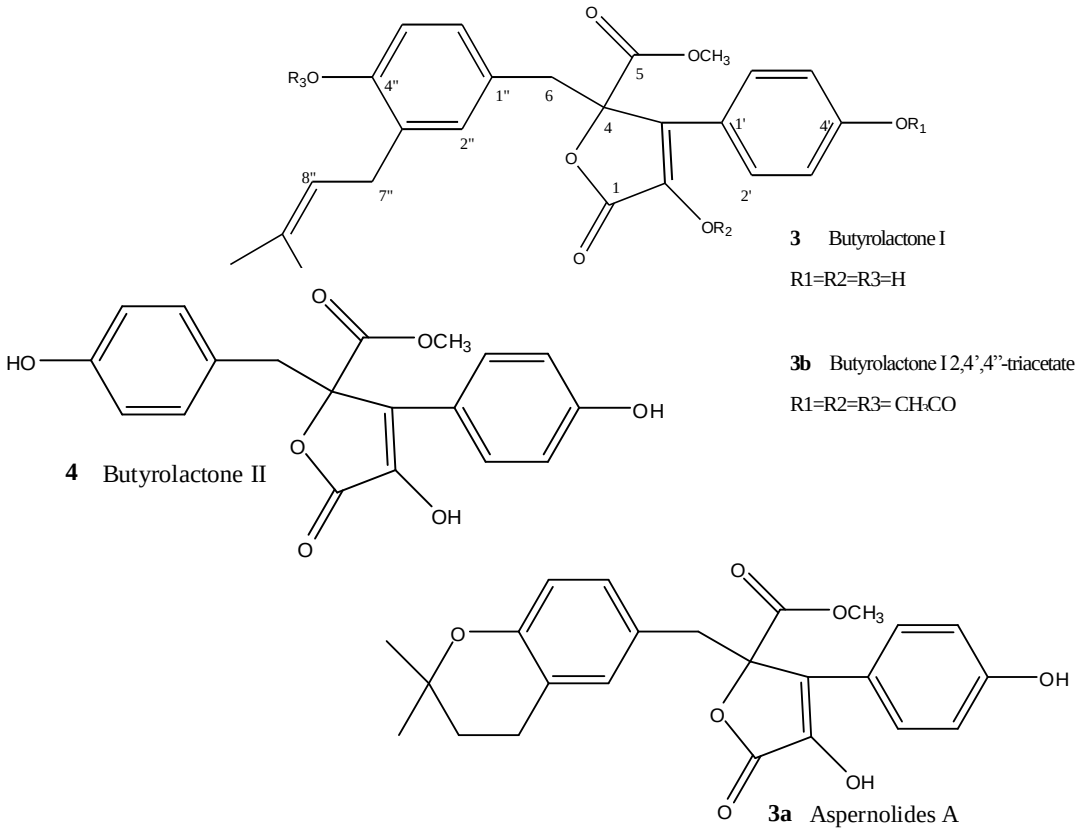


Figure 2. Structure of butyrolactone I derivatives

We investigated the inhibitory activities of butyrolactone I (**3**) and derivatives (**3a**, **3b**, and **3c**), and butyrolactone II (**4**) against α-glucosidase from *S. cereviseae*. Quercetin was used as a positive control based on the reports that it is a phenolic compound with a stronger inhibitory effect on α-glucosidase from *S. cereviseae* than

acarbose. The result was showed in Table 1, compound **3** was a potent inhibitor of the α-glucosidase with an IC_{50} of 52.17 μM. In contrast, compound **4**, which lack a prenyl side chain, exhibited less inhibitory activity. Converting the prenyl side chain to a dihydropyran ring in compound **3a** caused a significant decrease in the inhibitory activity. Hence, it was assumed that the prenyl side chain of compound **3** contributed to the inhibitory effect. However, the substitution of any hydroxyl group with an acetyl group in butyrolactone I led to a dramatic reduction in inhibitory activity in compound **3b.** Compound **3c**, which retained one OH-bond as an alpha hydroxy-lactone, showed significantly higher activity against α-glucosidase than compound **3b**. Based on the α-glucosidase inhibitory activities of compounds **3, 4**, SAR inference could be made. Compounds **3** and **3c** showed stronger activity than the others, wich suggested that the inhibitory effect of these butyrolactones was influenced by both the prenyl side chain and alpha hydroxy-lactone group. The influence of the prenyl side chain in butyrolactones was reported previously, however no SAR study of the α-glucosidase inhibitory activity of butyrolactone I derivatives has been reported before.

Table 1. Biologycal activities of butyrolactone derivatives from *A. terreus* MC751

Compound	IC_{50} (μM)[a]	
	Inhibition of α-glucosidase	Antioxidant
3	52.17±5.68	51.39±3.68
3a	175.18±5.95	47.55±3.08
3b	>300	n.d
3c	84.18±8.98	n.d
4	96.01±3.70	17.64±6.41
Quercetin	14.6±3.72	39.63±5.21

n.d not detected

[a]IC_{50} value are shown as mean ± S.D. from three independent experiment

The antioxidant activities of butyrolactone I (**3**), butyrolactone II (**4**), and derivatives (**3a**, **3b**, and **3c**) were evaluated in the DPPH free radical-scavenging assay. Compounds **3, 4,** and **3a** showed potential scavenging activity while compounds **3b** and **3c** did not. The antioxidant activity may originate from the phenolic groups.

Compound **4**, which lack a prenyl side chain, was the most powerful antioxidant. This result was indicated that a prenylated phenolic group in compound **3** decreased antioxidant activity. Cyclization of the prenyl group in

compound **3a** also decreased the antioxidant activity. In the case of compounds **3b** and **3c**, replacement of an OH group with an acetyl group dramatically decreased the antioxidant activity. Hence, it was concluded that the absence of a prenyl side chain increased the activity and acetylation appeared to be detrimental to the antioxidant activity.

The study of SAR of butyrolactone derivatives revealed that the prenyl side chain and hydroxyl group at lactone group in butyrolactone I has contribution for inhibitory α-glucosidase activity, however not for antioxidant activity. The compound **3** was found to be the most active compound as inhibitor of α-glucosidase (IC_{50}= 52.2 μM), while compound **4** was the most potential for antioxidant (IC_{50}= 17.6 μM).

Based on in-vitro study, we recommended that these metabolites: butyrolactone I and aspulvinone may have beneficial effect in managing the hyperglycemia effects. However further preclinical and clinical studies should be persuade before its pharmaceutical applications. This investigation may also provide as additional information on anti hyperglycemia properties of the secondary metabolites of *A. terreus* which are not reported earlier.

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