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Inhibitory Effect of Koji *Aspergillus terreus* on α-Glucosidase Activity and Postprandial Hyperglycemia

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Abstract: The compounds that could inhibit the activity of α-glucosidase are potentially used for antidiabetic by suppressing postprandial hyperglycemia. This research aimed to investigate the hypoglycemic activity in *A. terreus* koji extracted by ethyl acetate. The extracts was dissolved in methanol: water (1:4), followed by fractionations with n-hexane, methylene chloride and ethyl acetate. Each fraction was assayed for its activity against α-glucosidase. The active fraction was purified by column chromatography using silica gel and resin as adsorbent. The kopi extract showed potential as α-glucosidase inhibition with IC_{50} <10 μg mL^{-1} and showed combination of non-competitive and uncompetitive inhibition mode against α-glucosidase. Ethyl acetate fraction showed potential as inhibitor α-glucosidase with IC50 = 8.6 μg mL^{-1}. In animal experiment, active fraction (F10-4) of ethyl acetate fraction suppressed the increase of postprandial blood glucosidase level compare to the control. Thus it showed potential as α-glucosidase inhibitor and demonstrated depressed postprandial blood glucose level and may have potential use in the management of type 2 diabetes.

Key words: α-Glucosidase inhibitor, extract ethyl acetate koji *A. terreus*, anti-hyperglycemic effect

INTRODUCTION

At the present time estimated that 150 million people worldwide have diabetes and that will increase to 220 million by 2010 and with raising like the present in 2025 will 300 million, globally is non dependent insulin diabetes mellitus patient type 2 (Dong-Sun and Sang-Han, 2001; Collene *et al*., 2005). Postprandial hyperglycemia is one of the characteristic features of insulin resistance (Yamagishi *et al*., 2005) and play important role in the development of type 2 diabetes mellitus and complications associated with the disease, such as micro-and macro-vascular diseases (Cheng and Josse, 2004; Yuhao *et al*., 2005). Therefore, control of postprandial hyperglycemia is suggested to be important in the treatment of diabetes and prevention of cardiovascular complication.

One of the therapeutic approaches for decreasing of blood glucose rise after a meal is to retard the absorption of glucose by inhibition of carbohydrate hydrolyzing enzymes, such as α-amylase and α-glucosidase. α-Glucosidase is located in the brush-border surface membrane of intestinal cells and are the key enzymes of carbohydrate digestion, its specifically hydrolylized the α-glucopyranoside bond, releasing an α-D-glucose from the non-reducing end of sugar. The compounds that could inhibit the activity of α-glucosidase is potentially used for antidiabetic (Fujita and Tomohide, 2001; Collene *et al*., 2005; Kim *et al*., 2005). There are reports on established α-glucosidase inhibitors such as acarbose, miglitol and voglibose from microorganism (Yamagishi *et al*., 2005) and several α-glucosidase inhibitor have been recently screened and developed from natural source like nojirimicyn and 1-deoxynojirimycin from Rosell (*Hibiscus sabdariffa*) and leaves of *Morus bombysis* (Kim *et al*., 2005).

Aspergillus terreus is an especially prolific producer of secondary metabolites has biological activities such as: terrecyclodiol as antibiotic and antifungal (Almassi *et al*., 1996), lovastatin as anticholesterol (Schimel *et al*., 1998), asterriquinone as antineoplastich (Kaji *et al*., 1994) and aspulvinone and kodaistatin as inhibitor of hepatic glucose-6-phosphatase system (Vertesy*et al*., 2000). Our previous study on ethyl acetate extract of *A. terreus* koji shows significant activity as inhibitory of α-glucosidase (IC_{50} <10 μg mL^{-1}) (Triana *et al*., 2006). This result indicated this extract contained compound or compounds have potential as α-glucosidase inhibitor. In this study, we investigated the inhibitory effect and the pattern of

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inhibition mode of action of koji ethyl acetate extract (KEE). We also examined the effect of active fraction on postprandial blood glucose level after a meal in mice.

MATERIALS AND METHODS

This study conducted in Laboratory of Natural Products and Pharmaceutical and Laboratory of Pharmacology, Research Center for Chemistry-Indonesian Institute of Science on 2005-2006.

Microorganism and inoculum preparation: A wild-type strain of *A. terreus* is collected of Research Center for Chemistry-Indonesian Institute of Sciences, was used in the present study. It was maintained on Potato Dextrose Agar (PDA) slants, incubated for seven day at 30°C. For preparing a spore suspension, 5 mL of sterilized 0.1% Tween-80 solution was added to a well-sporulated slant of *A. terreus*. The concentration of the spore-suspension was measured using spectrometer and then adjusted to give transmitan 25% at wavelength 660 nm.

Sporulation medium: Sporulation medium contained (1 L); 15 g of glucose, 5 g of corn steep liquor (Sigma catalog No. C-4648), 5 g of oatmeal, 5 g of yeast extract (Difco) and 10 mL of trace element (1 g of $FeSO_4.7H_2O$; 1 g of $MgSO_4.7H_2O$; 200 mg of $ZnSO_4.7H_2O$; 100 mg of $CaCl_2.2H_2O$; 25 mg of $CuCl_2.2H_2O$; 56 mg of H_3BO_3; and 19 mg of $(NH_4)_6Mo_7O_{24}.4H_2O$ -per liter of solution). In a 250 mL Erlenmeyer flask media containing 50 mL sporulation medium, 2 mL spore suspensions was added. The flasks were shaken at 120 rpm for 2 day at 30°C.

Solid state fermentation in alumunium tray: Solid substrate (1 kg, Rice IR-42) washed twice with water, drained and autoclaved at 121°C for 15 min with addition water (1:1). After cooling, media was inoculated with 50 ml (5%) of *A. terreus* culture grew on sporulation medium. Media was thoroughly mixed, placed in four sterile alumunium tray (30×20 cm) and incubated for seven day at 30°C. For this study fermentation was conducted with 5 kg scale.

Extraction and isolation: Koji (5 kg) was macerated with EtOAc then evaporated. The dry extract of KEE (90 g) was dissolved in MeOH: H_2O (1:4), followed by fractionations with n-hexane, CH_2Cl_2 and EtOAc. Activity guided isolation was conducted by analysis the inhibitor α-glucosidase activity. Fractions showed potential activity (IC_{50}<10 μg mL^{-1}) were further purified by vacuum column chromatography.

Inhibition assay for α-glucosidase activity: The reaction mixture consisting 250 μL of 20 mM p-nitorpehenyl α-D-glucopyranoside (Sigma Chemical Co.), 495 μL of 100 mM phosphate buffer (pH 7.0) adding to flask contain 5 μL of sample dissolved in DMSO at various concentrations (3.125 to 25 μg mL^{-1}). The reaction mixture was pre-incubated for 5 min at 30°C, the reaction was start by adding 250 μL α-Glucosidase (0.075 unit) (EC 3.2.1.20 from Wako Pure Chemical Industry) incubation was continued for 15 min. The reaction stopped by adding 2 mL 0f 0.1 M Na_2CO_3. Activity of α-glucosidase was determined by measuring release of p-nitrophenol at 400 nm. Nojirimicin (Sigma N-7779) used positive control of α-glucosidase inhibitor.

Kinetics of inhibition against α-glucosidase: Inhibition modes of KEE against α-glucosidase activities were measured with increasing concentration of p-nitrophenyl α-D-glucopyranoside as a substrate in the absence or presence of koji extract at different concentrations. Inhibition type was determined by Lineweaver-Burk plot analysis of the data, which were calculated from the result according to Michaelis-Menten kinetics.

Animal experiment: In this study, eight-week-old strain DDY mice (25-30 g) from Lab. Pharmacology Research Center for Chemistry-Indonesian Institute of Science were used. Animal test placed in space with temperature 25±2°C. All animal tests fasted during 16 h before treatment. The animal test were divided into five treatment group: group I, mice administered with glucobay® 6, 5 mg kg^{-1} bb as positive control; group II, mice were administered with 14, 18 mg kg^{-1} of F10-4 ; group III, mice were administered with 28, 36 mg kg^{-1} of F10-4 bb, group IV, mice were administered with 56, 72 mg kg^{-1} F10-4 and group V, mice were only administered water as negative control. For posprandial effects, five minute after treatment the animal test were orraly administered sucrose (2 g kg^{-1}). Blood samples were taken after: 0, 15, 30, 60, 120 and 180 min. Administered sucrose time expressed as 0 minute at graph. Plasma glucose level was measured by One Touch Ultra (Lifescan, Jonhson-Jonhson Co.).

RESULTS AND DISCUSSION

In earlier study which fermentation was conducted at 1 kg scale (Triana *et al.*, 2006), the ethyl acetate extract showed has potential inhibition activity of inhibitory α-glucosidase (IC_{50} = 9.4 μg mL^{-1}) whereas nojirimicin has IC_{50} = 5 μg mL^{-1}. Inhibition mode of nojirimisin and KEE against α-glucosidase was investigated. Inhibition mode of KEE had a combination of non-competitive

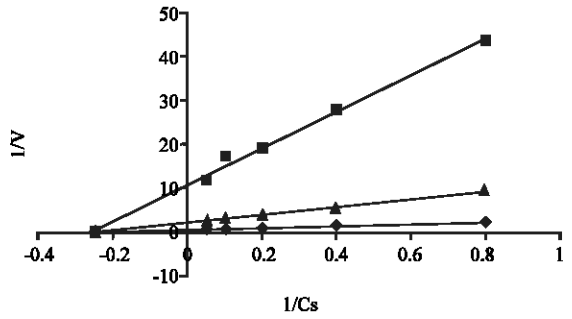

Fig. 1: Lineweaver-Burke plot of the reaction α-glucosidase in the presence of KEE (◆; no inhibitor, ■; 25 μg mL^{-1}, ▲; 12.5 μg mL^{-1})

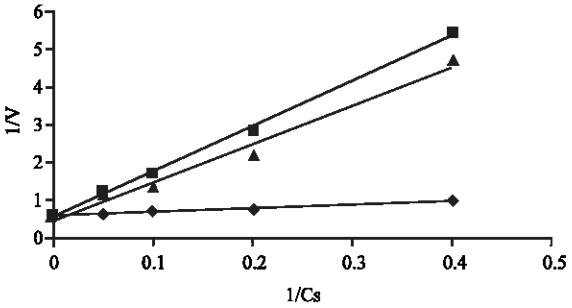

Fig. 2: Lineweaver-Burke plot of Nojirimicin (◆; no inhibitor, ■; 12.5 μg mL^{-1}, ▲; 3.125 μg mL^{-1})

and uncompetitive inhibition (Fig. 1) against yeast *S. cerevisiae* α-glucosidase, which in contrast with Nojirimicin that showed competitive inhibitory mode (Fig. 2) because the structure is aminisugars (sugar mimic) which similar with glucose. Combination of non-competitive and uncompetitive inhibition of KEE may have been due to the extract having more than one compound that has α-glucosidase inhibitory activity. Besides, the different inhibitory activities with nojirimicin may be caused by different structure. This result similar with inhibition mode of PBE (pine bark extract) which found a combination of non-competitive inhibition and uncompetitive inhibition against yeast *S. cereviceae* α-glucosidase (Kim *et al.*, 2005). This result indicates that KEE may be a novel type of inhibitor.

In this study we conducted 5 kg scale koji fermentation of *A. terreus*. Table 1 show that CH_2Cl_2 and EtOAc fraction showed potential α-glucosidase inhibitory activity. However since CH_2Cl_2 fraction was aimed to be used for lovastatin production as anticholesterol agent, therefore only EtOAc fraction was used for further antidiabetes study. EtOAc fraction was further fractionated by column chromatography (silica-gel, n-hexane: EtOAc) into ten major fraction 1-10. Each

Table 1: Yield fractionation and α-glucosidase inhibitory activity

Sample	Weight (g)	IC_{50} (μg mL^{-1})
Ethyl acetate extract	100.0	9.5
n-Hexane fraction	17.0	26.2
CH_2Cl_2 fraction	59.0	7.9
Ethyl acetate fraction	9.8	8.6
Water fraction	7.7	57.2
Nojirimicin	-	5.0

Table 2: Fractionation and α-glucosidase inhibitory activity of EtOAc fraction

Fraction	IC_{50} (μg mL^{-1})
F-1	NA
F-2	NA
F-3	NA
F-4	NA
F-5	NA
F-6	NA
F-7	NA
F-8	3.7
F-9	18.6
F-10	3.4

NA: No activity of α-Glucosidase inhibitory (IC_{50} > 100 μg mL^{-1})

Table 3: Fractionation and α-glucosidase inhibitory activity of F-10

Fraction	IC_{50} (μg mL^{-1})
F10-1	NA
F10-2	13.3
F10-3	4.0
F10-4/At	2.9

NA: No activity of α-Glucosidase inhibitory (IC_{50} > 100 μg mL^{-1})

fraction was tested for inhibitory activity against α-glucosidase. The Fraction F.10 showed most active with IC_{50} value is 3, 4 μg mL^{-1} (Table 2) and was further purified with column chromatography (resin HPD 600, EtOH;water) to give four fraction (Table 3). From *in vitro* assay, F10-4 (At) showed most potential activity, therefore we examined the effect of At on postprandial blood glucose level after a meal in mice.

In this study we used nojirimicin as positive control for *in vitro* assay however for animal experiment we used acarbose as positive control because it is a commercial available α-glucosidase inhibitor drug used for diabetes treatment. The effect of At on blood glucose levels after meal was examined using mice as animal experiment. Postprandial blood glucose levels of mice that were with administered At using three doses. Dosages given to the mice for postprandial blood glucose level experiment were calculated from IC_{50} value of *in vitro* assay (Miya *et al.*, 1968; Soemardji, 2004).

Postprandial blood glucose levels of the administered At (all doses) and acarbose were lower than those control (water). Blood glucose concentrations increased from 13.32 to 25.02 mmol dL^{-1} levels 60 min after a meal and decreased thereafter but not back to normal level until 180 min. However, the increase in postprandial blood glucose level was significantly suppressed on the At administered group (all doses, Fig. 3).

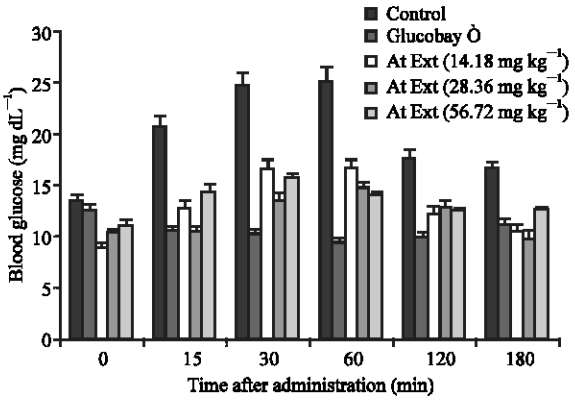

Fig. 3: Effect of postprandial blood glucose levels of At to control; glucobay®; Dose 1 (14.18 mg kg^{-1}); Dose 2 (28.36 mg kg^{-1}) and Dose 3 (56.72 mg kg^{-1}) were given simultaneously by oral administration to 7 week-old DDY mice after overnight (18 h) fasting. Blood sample were taken at 0, 15, 30, 60, 120, 180 min after the loading. Each point represents the mean±SE (n = 5)

Postprandial blood glucose levels of dose 1 (14.18 mg kg^{-1} w/w of mice) and dose 2 (28.36 mg kg^{-1} w/w of mice) show significant degradation but at dose 3 (56.72 mg kg^{-1} bb) rate of sugar relative higher at 15 min than dose 1 and 2, at 60 min after administrated rate of downhill and constant relative until 180 min. Difference of degradation of blood sugar rate between used dose not significant, this matter of possibility caused by difference of narrow dose range, so that cannot be determined by maximum dose of At. Reports from other studies showed that PBE was significantly suppressed the increase in postprandial blood glucose level in mice with dose 250 mg kg^{-1} (Kim *et al*., 2005) and PGF extract (*Punica granatum* flower) inhibits the increase of plasma glucose levels with dose 250-100 mg kg^{-1} (Li *et al*., 2005), therefore At showed activity at lower dose compare to PBE and PGF. Hence, At might content more potent anti diabetic compound.

In conclusion, KEE showed inhibitory activity on α-glucosidase by in vitro experiments. Result of fractionation KEE obtained F10-4 (At) showing most potential activity α-glucosidase inhibitory in vitro assay and show hypoglycemic activity on animal experiment. Currently attempts to purify the active compound from At are conducted, to understand the inhibitory mechanisms more clearly.

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