



## Isolation and $\alpha$ -Glucosidase inhibitory activity of endophytic fungi from mahogany (*Swietenia macrophylla* King) seeds

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**Abstract:** Diabetes mellitus is a metabolic syndrome characterized by hyperglycemia. The major goal in the treatment of this disease is to achieve normoglycemia. One of medication used is  $\alpha$ -glucosidase inhibitor that could reduce postprandial hyperglycemia with delay of digestion of carbohydrate. The ability of endophytic fungi to produce similar bioactive compounds to its host plant is potential source to get  $\alpha$ -glucosidase inhibitory compounds. This research was aimed to isolate the endophytic fungi from *Swietenia macrophylla* King seeds, and to evaluate the inhibitory activity of  $\alpha$ -glucosidase from fermentation culture of its isolate. Six endophytic fungi were isolated. Each isolate was fermented in submerged culture with Potato Dextrose Broth and yeast extract medium for 7 days, and then extracted with ethyl acetate and methanol.  $\alpha$ -Glucosidase inhibitory activity of those extracts were assayed by spectrophotometric method using microplate reader. Five ethyl acetate extracts showed better activity than acarbose with smallest IC<sub>50</sub> values was 73.64  $\mu$ g/mL. The most active extract showed competitive inhibition. Chemical analysis indicated that the  $\alpha$ -glucosidase inhibitor was flavonoid group.

**Keywords:**  $\alpha$ -glucosidase inhibitor; diabetes mellitus; endophytic fungi; mahogany; *Swietenia macrophylla* King.

### Introduction

Diabetes mellitus is a metabolic syndrome characterized by hyperglycemia and abnormalities in carbohydrates, fat, and protein metabolism that result from defects in insulin secretion, insulin sensitivity, or both (Wells et al. 2006). Diabetes mellitus is one of major health problems in Indonesia. According to the World Health Organization, the prevalence of diabetes mellitus in Indonesia will grow from 8.4 million people in 2000 to 21.3 million people in 2030 (Wild et al. 2004).

Goals of therapy in diabetes mellitus are reducing symptoms of hyperglycemia to prevent later complication, intensive therapy for associated cardiovascular risk factors, and improving quality of life (Triplitt et al. 2005). At present,  $\alpha$ -glucosidase inhibitors are the most common oral agents used to decrease postprandial hyperglycemia, since they can decrease glucose intake with low hypoglycemic effect

(Hanefeld and Schaper 2007). In addition, many researches showed that some natural product from various medicinal plants and microorganisms have potencies as  $\alpha$ -glucosidase inhibitors (Elya et al. 2011; Suthindhiran et al. 2009). One of them is *Swietenia macrophylla* King. Methanolic extract of seeds of *Swietenia macrophylla* King. has hypoglycemic effect in both aloxan and streptozotocin induced diabetic rats (Maiti et al. 2008).

Exploration medicinal plants as source of bioactive compounds would decrease natural diversity that cause extinction of plants species. Thus, another alternatives were needed as source of bioactive compounds. Production of bioactive compounds can be increased by biotechnology of endophytic fungi in order to meet demands while keeping biodiversity and sustainable ecosystem (Onifade 2007). Fermentation of endophytic microbes to produce bioactive compounds would give more advantages since it would be fast, reproducible,

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unlimited and weather/season independent (Dompeipen et al. 2011).

## Materials and methods

### *Isolations of endophytic fungi*

*Swietenia macrophylla* King. seeds collected from Bogor Botanical Garden, West Java were identified by Herbarium Bogoriensis Indonesia Institute of Science. Seeds of *S. macrophylla* King were rinsed with tap water before surface sterilised. Seeds were thoroughly surface sterilised by soaking them in 70% ethanol for 3 minutes, 5.25% NaOCl solution for 5 minutes, and 70% ethanol for 30 seconds. Then, samples were air-dried on sterile tissue paper inside the biological safety cabinet. Samples were plated on three different media: Corn Meal Malt Agar, Potato Dextrose Agar and Water Agar containing 0.05% w/v chloramphenicol. Cultures were incubated for 5-21 days in 27°C. The endophytic fungi were purified on PDA media and incubated for 5-7 days. Then, those isolates were transferred to PDA slant agar as working culture and stock culture.

### *Examination of endophytic fungi colonies*

In this research we performed morphological examination. Colonies were identified based on morphological characteristics using macroscopic and microscopic methods. Macroscopic method was made by observing colony morphology, color and reverse colony color. Microscopic method was made by staining with lactophenol cotton blue using cellotape flag preparations and observing it under microscope (Ellis et al. 2007).

### *Extraction of fermentation culture*

Endophytic fungi were transferred to 500 mL PDY broth in 1000 mL Erlenmeyer flask, and agitated at 150 rpm for 7 days at room temperature. Fermentation culture was divided into two parts. The first part was mixed with ethyl acetate using vortex mixer for 5 minutes then centrifuged at 3000 rpm for 15 minutes and the supernatant was used for inhibition assay of  $\alpha$ -glucosidase activity. The second part was centrifuged at 3000 rpm for 15 minutes and the

supernatant was used for inhibition assay of  $\alpha$ -glucosidase activity. Its biomass was mixed with methanol using vortex mixer for 5 minutes then centrifuged at 3000 rpm for 15 minutes, and the supernatant was used for inhibition assay of  $\alpha$ -glucosidase activity. Each methanol and ethyl acetate extract was evaporated in rotavapor and dried in vacuum oven (30°- 40°C) and each water extract was freeze-dried.

### *Inhibition assay of $\alpha$ -glucosidase activity*

$\alpha$ -Glucosidase recombinant *S. cerevisiae*  $\alpha$ -glucosidase (Sigma-Aldrich, USA) was dissolved in phosphate buffer (pH 6.8) containing 0.2% bovine serum albumin until final concentration 0.05 unit/mL. p-Nitrophenyl  $\alpha$ -D-glucopyranoside (PNPG) (Sigma-Aldrich, USA) as substrate was dissolved in phosphate buffer until final concentration 10 mM. Extracts were dissolved in DMSO, then diluted in the phosphate buffer. The reaction mixture consisting 2-10  $\mu$ L sample solution was added with 55-63  $\mu$ L phosphate buffer pH 6.8 and 10  $\mu$ L 10 mM PNPG. Then, the reaction mixture was incubated for 5 minutes. Enzymatic reaction is started by adding 25  $\mu$ L enzyme solutions (0.05 unit/mL) to mixture and incubation was continued for 30 minutes. Enzymatic reaction is stopped by adding 100  $\mu$ L 200 mM Na<sub>2</sub>CO<sub>3</sub>. The absorbance at 405 nm was measured using a microplate reader. Acarbose (Actavis, Indonesia) was used as positive control of  $\alpha$ -glucosidase inhibitor.

Inhibition percentage was calculated using formula:

$$\% \text{ inhibition} = \frac{(C - S)}{C} \times 100\%$$

which S is absorbance of sample and C is absorbance of blank. IC<sub>50</sub>, the concentration of extract inhibiting 50% of alpha-glucosidase activity under the stated assay conditions, was determined by using linear regression, with formula  $y = a + bx$ , where Y is inhibition percentage and X is sample concentration, then the formula:

$$IC_{50} = \frac{50 - a}{b}$$

### Enzyme kinetic assay

Enzyme kinetic assay was measured with increasing concentration of PNPG as a substrate in the presence or the absence of the extract (Dewi et al. 2007). Mode of inhibition was determined by Lineweaver-Burk plot analysis of the data calculated following Michaelis-Menten kinetic.

### Phytochemical test

In this research we performed phytochemical analysis by using some identification reagents, i.e. Mayer, Dragendorff, and Bouchardat for alkaloid; Shinoda and Wilson Toubock reaction for flavonoid; gelatin test, gelatin-salt test, and ferrous (III) chloride for tannin; Molisch reaction for glycoside; Honeycomb froth test for saponin; Borntrager reaction for antraquinone; Lieberman-Buchard for terpenoid. The analysis also was performed with TLC using specific spray reagents. Spray reagents used were Dragendorff for alkaloid,  $AlCl_3$  for flavonoid, and  $FeCl_3$  for tannin (Waksmundzka-Hajnos et al. 2008).

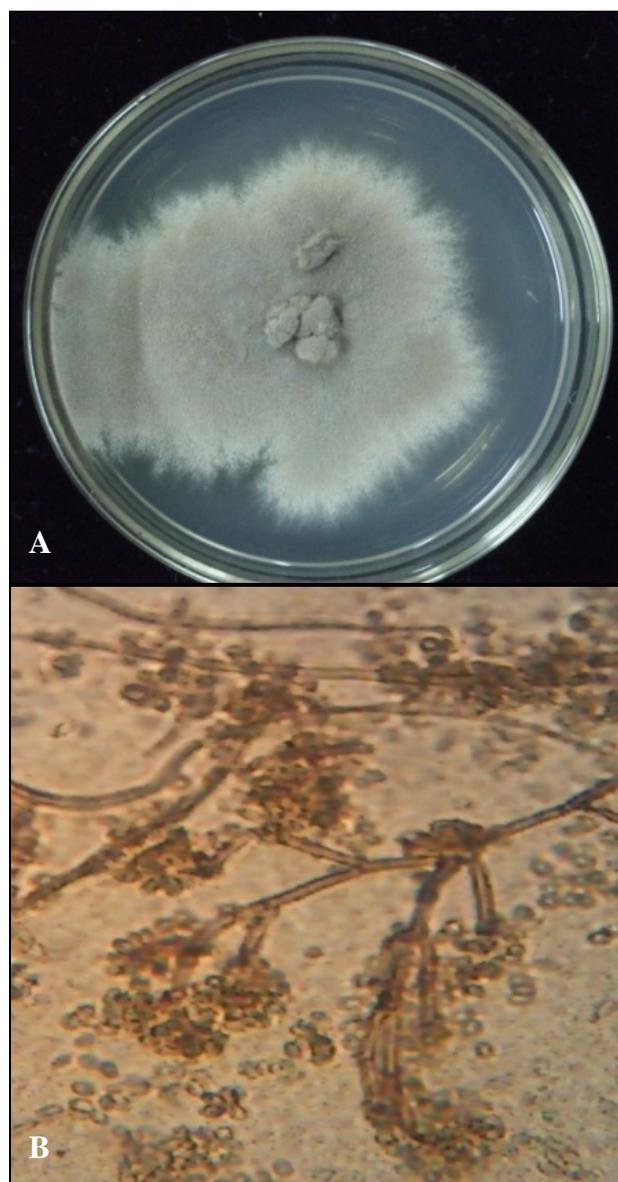
## Result and discussion

Endophytes are able to produce similar bioactive compounds to its host plants (Tan and Zou 2001). Natural products from endophytic fungi have a broad spectrum of biological activity. They can be grouped into several categories, there are alkaloids, steroids, terpenoids, isocoumarins, quinones, phenylpropanoids and lignans, phenol and phenolic acids, aliphatic metabolites, lactones (Zhang et al. 2006). *Aspergillus* A isolated from the marine-derived fungus *Aspergillus aculeatus* is proved to have good  $\alpha$ -glucosidase inhibitory activity (Ingavat et al. 2009). Koji and Nojirimycin were isolated from some species of fungi and bacteria. They are known as potential  $\alpha$ -glucosidase inhibitors (Dewi et al. 2007; Borges et al. 2006).

Endophytic fungi are extremely common and highly diverse microorganisms that live within plant tissues, but usually remain asymptomatic (Faeth and Fagan 2002). Endophytic fungi can be isolated on appropriate

media. Routine mycological media are suitable for isolation and for subculturing for identification of endophytic fungi. Malt extract agar is most commonly used and sometimes in combination with yeast extract. Antibiotics are often used for primary isolations for prevent contamination. Water agar is weak media but it is used for isolations to reduce contamination, although many fungi produce more diffuse, spreading, and less recognizable colonies (Stone et al. 2004).

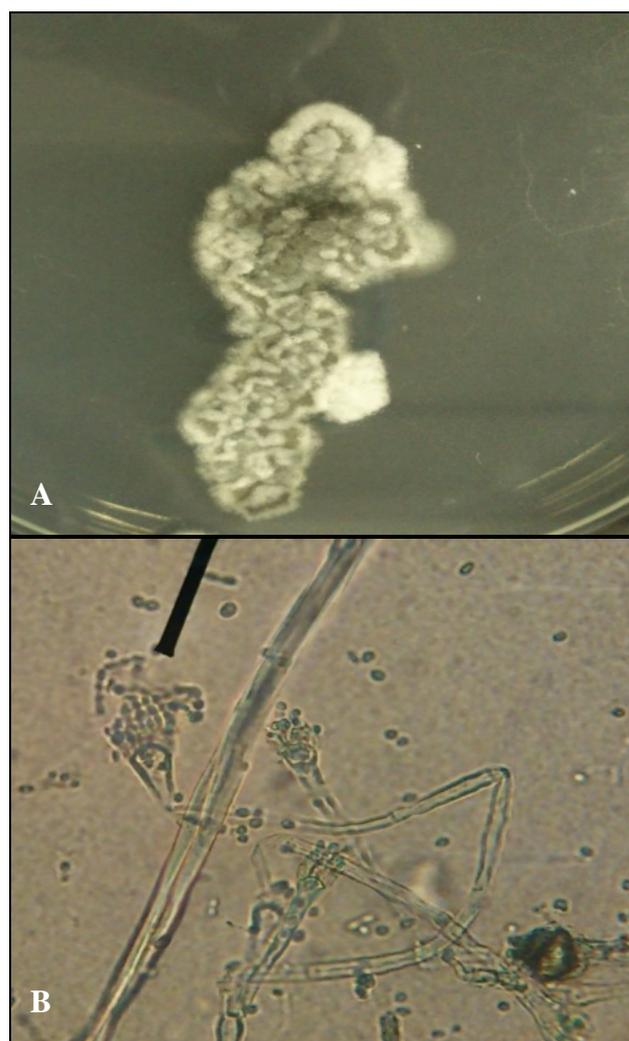
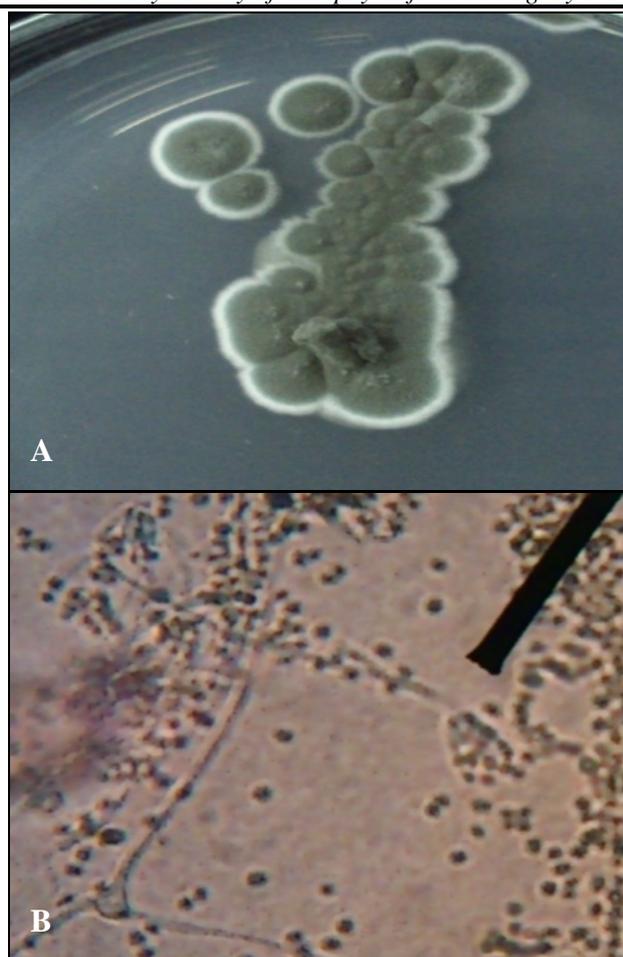
In this study, 6 endophytic fungi were isolated from *S. macrophylla* King. seeds. Characteristic of fungi isolates can be seen in Table 1.



**Figure 1:** Isolate CMM4B from seeds of *S. macrophylla* King. (A) at PDA medium (B) microscopic (400x)

**Table 1:** Characteristic of endophytic fungi isolates.

No Isolate code	Morphology and color of colony in PDA medium
1 CMM4B	greyish brown, brown to black reverse, smooth surface, becoming powdery, produced a brown diffusible pigment
2 CMM11A	green with white mycelia, white to yellow reverse, rough surface
3 CMM19A	green with white periphery, smooth surface, becoming powdery, light-brown reverse, produced a green diffusible pigment
4 PDA1A	white, cotton-like surface, light-brown reverse
5 WA6B	white to greenish, white to yellowish reverse, cotton-like surface
6 WA8A	dark-green, becoming powdery, yellowish-white reverse, white periphery

**Figure 2:** Isolate CMM11A from seeds of *S. macrophylla* King. (A) at PDA medium (B) microscopic (400x)**Figure 3:** Isolate WA8A from seeds of *S. macrophylla* King. (A) at PDA medium (B) microscopic (400x)

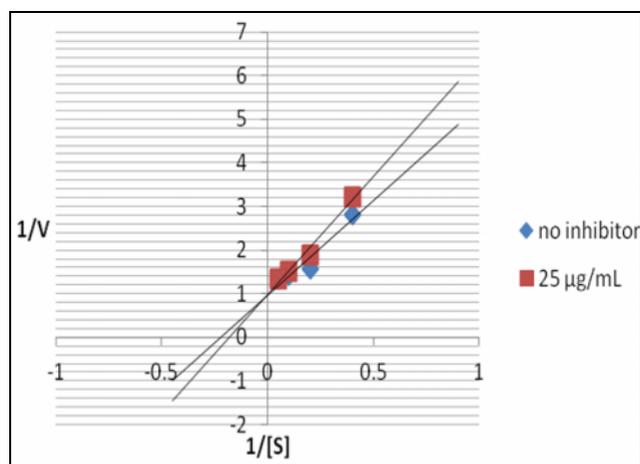
In this assay, we found % inhibition of water extract and methanolic extract at 1000  $\mu\text{g/mL}$  less than ethyl acetate extract. Thus,  $\text{IC}_{50}$  values both of water extract and methanol extract were more than 1000  $\mu\text{g/mL}$ . It is suggested that  $\alpha$ -glucosidase inhibitor is more soluble in semi polar solvent. It was similar to Dewi, et al (2007) study which had been purified Aspergillusol A from ethyl acetate fraction of *Koji A. terreus*. Therefore, we only investigated ethyl acetate extract.

The inhibitory activity of the ethyl acetate extract of endophytic fungi from *S. macrophylla* King. against yeast  $\alpha$ -glucosidase is shown in Table 2. We found 5 ethyl acetate extract less than  $\text{IC}_{50}$  value of that acarbose and the smallest  $\text{IC}_{50}$  value was 73.64  $\mu\text{g/mL}$  from CMM4B isolate.

**Table 2:** IC<sub>50</sub> value of crude ethyl acetate extract of fermentation culture and acarbose.

No	Isolates	IC <sub>50</sub> ( $\mu$ g/mL)
1	CMM4B	73.64
2	CMM11A	352.93
3	CMM19A	115.35
4	PDA1A	230.58
5	WA6B	229.67
6	WA8A	172.52
7	Acarbose	117.06

Mode of inhibition of the most potent extract was investigated by using Lineweaver-Burk plot. Lineweaver-Burk plot showed competitive inhibition (Figure 4). Constanta of Michaels (Km) also showed competitive inhibition type. Competitive inhibitor increase Km values but Vmax values remain constant (Rodwell and Kennelly 2003).

**Figure 4:** Lineweaver-Burk plot of  $\alpha$ -glucosidase inhibition of ethyl acetate extract of CMM4B isolate.

IC<sub>50</sub> of five ethyl acetate extracts are smaller than acarbose. This is promising for advance study to isolate the active substances for development of new inhibitor of  $\alpha$ -glucosidase. Phytochemical identification of the most potent extract using spot test showed that the extract contents flavonoid. It was supported by result of TLC test of the extract using hexane-ethyl acetate (8:2) as mobile phase which showed some spots after sprayed by AlCl<sub>3</sub> solution.

## Conclusion

Isolation of endophytic fungi from seeds of *S. macrophylla* King. has been done. We succeeded in isolating six endophytic fungi colonies. In vitro assays of  $\alpha$ -glucosidase inhibitory activity from their fermentation culture extracts showed five ethyl acetate extract had better activity than that acarbose. Ethyl acetate extract of CMM4B fermentation culture was the most potent extract with IC<sub>50</sub> value of 73.64  $\mu$ g/mL. Mode of inhibition from CMM4B ethyl acetate extract was competitive inhibitor. Flavonoid was suggested responsible for the  $\alpha$ -glucosidase inhibitory activity.

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