The Alpha-Amylase Inhibition Potential of Endophytic Fungi from Indonesian Bay Leaves (*Eugenia polyantha* WIGHT.)

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Abstract: Indonesian people use bay leaves as spices in local culinary and as traditional medicine, particularly to treat diabetes. The problems with the mass production of antidiabetic drugs from bay leaves can be solved by utilizing endophytic fungi as an alternative source for antidiabetic compounds. This study aimed to isolate endophytic fungi from bay leaves and identify their antidiabetic activity through the in vitro inhibition of alpha-amylase. The leaves were processed on potato dextrose agar media, and five isolates were grown in an agar medium. The fermentation used a potato dextrose yeast medium that was left for five days on an orbital shaker at room temperature. The crude was extracted using ethyl acetate solvent. In the in vitro alpha-amylase inhibition test, the antidiabetic assay used the ethyl acetate extract of the endophytic fungi. The inhibition percentage was calculated from the absorbance value read by a microplate reader. All isolates inhibited alpha-amylase activity, but only three of them had high inhibition percentages (14.385%, 12.849%, and 39.246%). As a conclusion, the endophytic fungi isolated from bay leaves are potential as an alternative source for the production of secondary metabolites to cure diabetes.

1 INTRODUCTION

Eugenia polyantha WIGHT. is one of the plants in Indonesia that are commonly used as spices in many local dishes. Empirically, this plant, especially the leaf, has been widely used as medicine since the ancient time. People believe that the leaves can cure some diseases, including diabetes (Aminov, 2010; Lelono & Tachibana, 2013; Elya *et al*., 2015; Murugan *et al*., 2017).

Diabetes is a chronic disease caused by insufficient production of insulin hormones by the pancreas. It is characterized by the elevation of blood sugar in the body. It can trigger some chronic metabolite syndromes, such as cardiovascular diseases and kidney disorders. According to the statistical data reported by World Health Organization (2016), in 2012, diabetes directly caused 1.5 million deaths, and another 2.2 million deaths were caused by diabetes-related diseases, such as cardiovascular and kidney disorders. Furthermore, in 2014, an estimated 422 million adults over 18 years of age world wide had diabetes. The Indonesian Ministry of Health reports that 12 million people in Indonesia have diabetes. Moreover, WHO reveals a slight increase in the number of diabetic people in Indonesia from 1996 to 2014 (WHO, 2016).

There are several preventive and curative ways to reduce the number of diabetic people. For prevention, WHO designs early detection methods, such as blood glucose measurements, oral glucose measurements, and HbA1c test. This organization also introduces new preventive methods, namely telemedicine and mobile phone-assisted intervention to reach remote areas. In 2014, the government of Senegal launched a diabetes monitor application called mRamadan, which received support from WHO. This approach improves the diabetes management in remote areas. Besides prevention, diabetes requires treatments that focus on the reduction of blood glucose level. Some of the commonly used medicines are insulin (by injection or oral administration), metformin, glibenclamide, and sulfonylurea (Serrano-Cinca *et al*., 2005; WHO, 2016).

Nowadays, people still believe that plants can be used as alternative medicines. As the results, bay leaves remain as an alternative source to cure diabetes. The high price of antidiabetic medicines

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contributes to this preference. Most of them are not affordable for some people from low- to middle-income families, limiting their options to herbal medicines. The high expectation of the therapeutic benefits of bay leaves triggers herbal medicine companies to commercialize any drugs made of bay leaves. However, they have to face a major obstacle, that is finding the area to plant *E. polyantha* WIGHT. The utilization of microbes in the production of metabolites to cure diabetes experiences a corresponding situation. The potentially explorable microbes, in this case, are endophytes. Endophytes are groups of microbes that live inside plant tissue and have a symbiotic relationship with the plants. They are also responsible in the biosynthesis of secondary metabolitesin the host plants (Strobel & Daisy 2003; Staniek *et al*., 2008; Mishra *et al*., 2014; Pimentel *et al*., 2011).

Based on the previous information, this study aimed to investigate the potential of endophytic fungi for producing secondary metabolites that could inhibit alpha-amylase.

2 MATERIALS AND METHOD

2.1 Materials

This study used fresh bay leaves from Bekasi, West Java, Indonesia. It also used some media and reagents for the experiments. The endophytic fungi isolation used 70% ethanol, 5.3% NaOAc, Chloramphenicol (Generik, Indonesia), Potato Dextrose Agar or PDA (Merck, USA), aquadest. The Potato Dextrose Yeast or PDY (Merck, USA) and ethyl acetate solvents were also used for the fermentation and extraction process. As for the alpha-amylase inhibition assay, the materials were alpha-amylase enzyme from *Bacillus* sp. (Sigma-Aldrich, USA), Phosphate Buffer Saline or PBS (Sigma-Aldrich, USA), soluble starch (Sigma-Aldrich, USA), acarbose (Generik, Indonesia), and the Lugol's reagent.

2.2 Methods

This study consist of several steps, namely endophytic fungi isolation, fermentation and secondary metabolites extraction, and alpha-amylase inhibition assay as the last procedure.

2.2.1 Endophytic Fungi Isolation

This step began with leaf surface sterilization as described in Widowati *et al*. (2016). The sterilized leaves were then cut into several pieces (1 cm x 1 cm), put into a sterile PDA medium, and incubated at room temperature for seven days.

2.2.2 The Fermentation and Extraction of Secondary Metabolites

The fermentation was based on the protocol introduced by Suciatmih *et al*. (2011) with some modifications. Two pieces of endophyte isolates (1 cm x1 cm) were added to 20 ml of PDY medium and incubated in a shaking incubator at room temperature for seven days at 170 rpm. Afterward, the metabolites were harvested by extracting the fermented medium. The extraction involved the addition of 1 volume of ethyl acetate into the fermented medium that had already separated from mycelium. The extract was then dissolved in PBS for 500 ppm dosage.

2.2.3 The Inhibitory Activity of Alpha-Amylase Assay

This process followed the inhibition assay for alpha-amylase described in Kellogg *et al*. (2014). It used the extract as the samples, acarbose as the positive control, the uninhibited enzyme as the negative control, and the extracts or acarbose without enzyme as blanks. For the samples and the positive control, this research used 96-well plate filled with 35 ul of the extract or acarbose and 5 ul of the starch substrate. The plates were then incubated at 37^0C for 5 min. Afterward, 10 ul of the alpha-amylase enzyme solution was added to each well and incubated at 37^0C for 10 min. The addition of 150 ul of Lugol's solution to each well would stop the reaction. Then, the inhibition percentage was calculated according to the absorbance value read by a microplate reader at a wavelength of 595 nm. The calculation of the inhibitory activity of each sample used the formula (1)

$$\%\ Inhibitory\ Activity = \frac{A_{con} - (A_{ext} - A_{blank})}{A_{con}} x100\% \quad (1)$$

A_{con} is the absorbance of the uninhibited enzyme, A_{ext} is the absorbance of the enzyme treated with the extract, and A_{blank} is the absorbance of the extract with the substrate (no presence of enzyme).

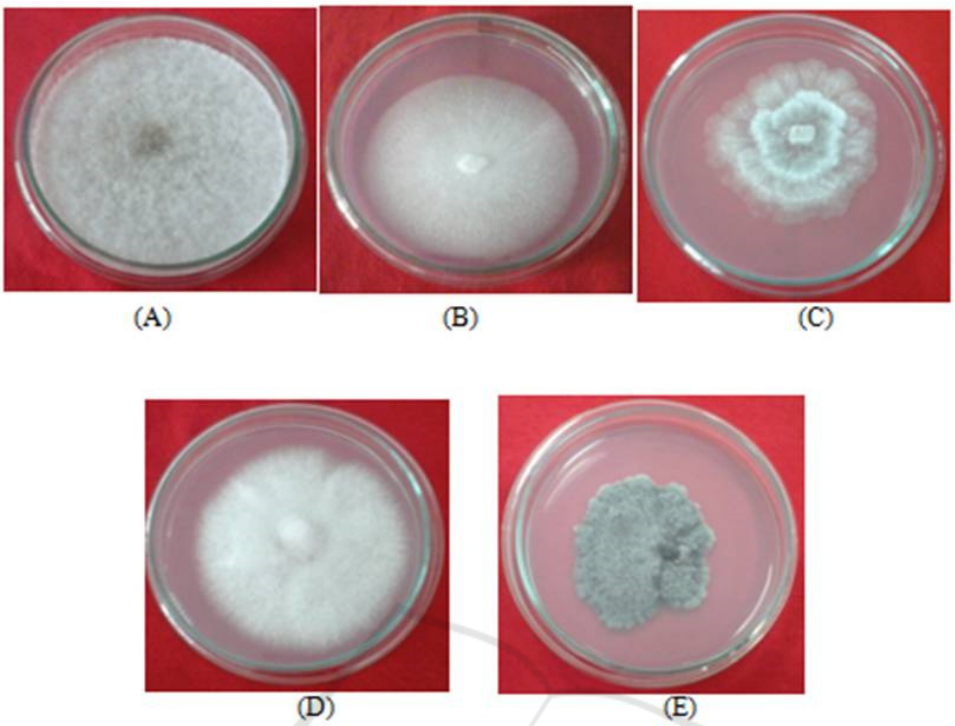

Figure 1. The isolates of endophytic fungi from Indonesian bay leaves: (A) KSP-O1, (B) KSP-02, (C) KSP-03, (D) KSP-04, and (E) KSP-05

2.2.4 Data Analysis

The analysis results were the average value (in mean±SD) of the triplicate assay.

3 RESULTS AND DISCUSSION

The term endophyte defines the community of microbes living inside plant tissue without damaging their host. These organisms are reported to be responsible for the secondary metabolic biosynthesis of their host plants (Strobel & Daisy 2003; Strobel 2006; Staniek *et al*., 2008; Alvin *et al*., 2014; Zhang *et al*., 2012). Therefore, the microbes produce some compounds that can be found in the host plants (Qiu *et al*., 2015; Zhang *et al*., 2012; Tan & Zou 2001).

The isolation of microbes from their host is the most difficult procedure in endophyte research due to the risk of contamination. As a preventive measure, some antibiotics were introduced to the isolation medium. This study used Chloramphenicol to inhibit the presentation of bacteria. The isolation commenced with placing the part of the plants' organ on the agar medium. Afterward, this medium was incubated for several days. This research obtained five endophytic fungi after seven days of incubation (Figure. 1).

The secondary metabolites were cultivated in a liquid medium using the liquid fermentation method. The harvested metabolites were collected through ethyl acetate extraction. This study used ethyl acetate instead of other solvents because the targetted compounds were flavonoids. The other reason for choosing the solvent was polarity. Ethyl acetate is a semipolar solvent, meaning that it attracts polar to semi-polar metabolites (Haque *et al*., 2005). Flavonoid is polar and, therefore, removable by ethyl acetate.

The antidiabetic activity of extracts from plants or microbes can be measured *in vitro* and *in vivo*. There are two types of assay commonly used in *in vitro* experiments, namely β-glucosidase and α-amylase inhibition. Analyzing the inhibition of these enzymes is necessarybecause they induce the absorption of glucose and the associated postprandial hyperglycemic spike (Kellogg *et al.,* 2014; Sahani *et al*., 2017; Ruzieva *et al*., 2017). Such inhibition is a strategy in diabetes management as it can control the serum glucose level.

Table 1. The Alpha-amylase Inhibitory Potential (% Control) of Endophytic Fungi Crude Extract and Acarbose

Samples	Percentage of inhibition (% ± SD)
KSP-01	14.38 ± 1,95
KSP-02	12.85 ± 3,91
KSP-03	6,94 ± 5,38
KSP-04	4,42 ± 2,51
KSP-05	39,24 ± 3,28
Acarbose	46.50 ±1.42

This study only performed the inhibition assay for alpha-amylase. Alpha-amylase is an enzyme that hydrolyzes carbohydrate into glucose, and in advance, it is easily absorbable (Kellogg *et al*., 2014). Most of this enzyme is present in saliva, and, as aconsequence, the serum glucose level becomes higher easily because the hydrolyzation of carbohydrate already begins in the mouth (Sahani *et al*., 2017). The inhibitio n of alpha-amylase activity decelerates the conversion of starch into glucose and effectively reduces glucose absorption (Ruzieva *et al*., 2017; Sahani *et al*., 2017). Based on the data presented in **Table** 1, each isolate of the endophytic fungi inhibited the enzyme at different rates. Among the five isolates, KSP-4 had the lowest activity. On the contrary, KSP-5 had the highest percentage of inhibitory activity, but it was lower than acarbose (Figure.2).

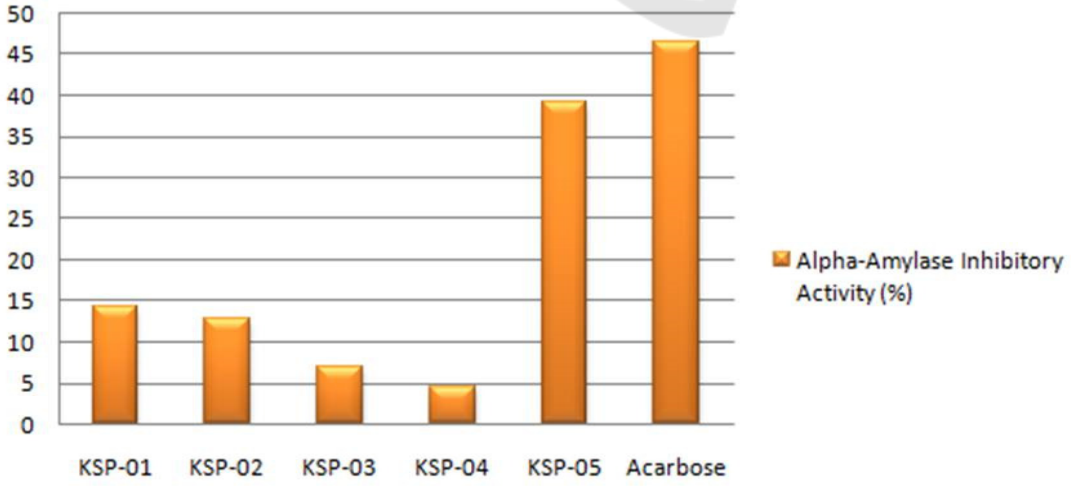

Figure 2. The Graphic of the Alpha-Amylase Inhibitory Activity (%) of Endophytic Fungi Isolated from the Indonesian Bay Leaves and Acarbose

4 CONCLUSIONS

Nowadays, endophytes become increasingly attractive to observe particularly due to the difficulty of finding a large area to grow medicinal host plants for product commercialization. One of the chronic diseases that causes high amount of mortality is diabetes. Diabetes can be handled with prevention and medication. Reducing blood glucose level is the effective management in increasing the survival rate of diabetic people. This study showed that the endophytic fungi from one of the Indonesian medicinal plants could inhibit the activity of alpha-amylase. Further investigation is needed to optimize the extraction to obtain the lowest concentration with the greatest inhibitory activity and to identify the in vitro inhibitory ability of endophytic fungi against the alpha-glucosidase enzyme.

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