Phytochemicals and antidiabetic activity of *Eusideroxylon zwageri* stem bark collected from East Kalimantan, Indonesia

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Abstract. *Eusideroxylon zwageri* (Lauraceae), a tropical tree species known as ulin or borneo iron wood and traditionally used for the treatment of diabetes in the Ethnic of Kutai. Plant extract was prepared by maceration using ethanol. The plant extract was evaluated its DPPH and superoxide radicals scavenging activity, the inhibition on α-glucosidase and α-amylase activity as antidiabetic potential and the analysis of the total phenolic, total flavonoids and proanthocyanidin contents. The ethanolic extract of the stem bark was 8.62% on the dry weight basis. The IC₅₀ values of antioxidant activity of the extract in DPPH and superoxide radical scavenging mechanisms were 44.90 µg/ml and 30.47 µg/ml. In antidiabetic assay, the *E. zwageri* stem bark extract showed IC₅₀ value 58.45µg/ml in α-glucosidase inhibition, and 9.04 µg/ml in α-amylase inhibition. Quercetin, an antidiabetic activity-having flavonoid, displayed IC₅₀ values 2.00 µg/ml and 4.04 µg/ml in α-glucosidase and α-amylase inhibitory assays. In phytochemical assay, the extract had 31.28 GAE/g extract (mg), 30.48 CE/g extract (mg) and 183.3 PE/g extract (mg) for the total phenolic, total flavonoid and total proanthocyanidin contents. The limited reports of *E. zwageri* indicated the needs to search the active compounds from plant as potential antidiabetic agents by considering plant conservation status.

1. Introduction
Diabetes mellitus (DM) is one of the chronic diseases associated with metabolic disorders of the body. There are two main categories of diabetes mellitus, type 1 and type 2. Type 1 diabetes, or diabetes with insulin dependence, is a condition in which the body has a low ability or even not able to secrete insulin to prevent metabolic disorders. Type 2 diabetes, or diabetes without insulin dependence, is a condition in which the body experiences insulin deficiency or insulin resistance to regulate blood sugar levels for optimal needs for the body. The impaired metabolism followed by the release of excessive free radicals will cause imbalance generally lead to oxidative stress which is the main factor associated with the severity and death in diabetes. Diabetes mellitus is a major cause of disability, figuring among the top ten killers worldwide [1,2].

An approach to diabetes therapy is through the inhibition mechanism of enzymes that play an important role in the absorption of glucose in the human metabolism, such as α-glucosidase and α-amylase enzymes. Some studies have illustrated the potential for inhibition of the enzyme α-
glucosidase by plants and also its effect on blood sugar levels [3]. Inhibition of the α-glucosidase enzyme also shows a slowing in the absorption of carbohydrates [4]. Therefore, α-glucosidase and α-amylase inhibitors are potential for use in the treatment of type 2 DM and other diseases associated with hyperglycemia. Plants are a potential source of natural medicine as there are currently a number of commercial drugs derived from plants. The search for natural ingredients derived from plants for enzyme inhibitors that play a role in glucose absorption has been the focus of intensive research [5].

Indonesia is one of the countries having a richness of plant diversity including medicinal plants. The richness is also supported by the potential of local wisdom that grows and develops in various ethnic groups residing in Indonesia. The objectives of this study were to evaluate the antidiabetic activity of the ethanol extract of Euordexylon zwageri stem bark through the inhibition mechanism of α-glucosidase and α-amylase enzymes and to investigate the DPPH and superoxide free radical scavenging activities.

2. Materials and Methods

2.1. General
Plant sample was collected from Kutai Kartanegara (Kutai ethnic) districts in July 2016. The sample was identified by taxonomist at the Laboratory of Dendrology, Faculty of Forestry Mulawarman University. Plant specimens were stored in the same laboratory.

Chemicals and biological enzymes used include ethanol, n-hexane, chloroform, ethyl acetate, 1,1-diphenyl-2-picrylhydrazil (DPPH), sodium hydrogen phosphate, disodium hydrogen phosphate, β-nicotinamide adenine dinucleotide, nitroblue tetrazolium, phenazine metosulfate, α-glucosidase enzyme, α-amylase enzyme, nitrophenyl α-glucopyranoside, dinitrosulfanilic acid, dimethyl sulfoxide, acarbose, ascorbic acid, starch, gallic acid, Folin-Ciocalteau reagents, sodium carbonate, aluminum chloride, ascorbic acid, quercetin, pelargonidin and other reagents were purchased from chemical companies.

2.2. Preparation of Extracts
The process of extraction was conducted by using maceration method. Approximately 50 g of E. zwageri stem bark were soaked in ethanol for 2 x 24 hours. The maceration was repeated 2 times according to the response of the plant material to the solvent. The sample solution was filtered by Whatman filter paper no. 2. The resulting solution of the filter was concentrated with a rotary evaporator at the temperature of 40 °C. The condensed extract obtained was then stored in a vacuum oven at 35 °C until a solid extract was obtained.

2.3. Antioxidant activity

2.3.1. DPPH radical scavenging capacity. The antioxidant activity assay used in this study was based on Shimizu et al method [6]. The test was performed using a UV-Vis spectrophotometer at room temperature (25 °C) with a wavelength of 517 nm and using DPPH solution (1,1-diphenyl-2-picrylhydrazyl). A total of 33 µl of plant extract (1.5–200 µg/ml) in DMSO were added to the cuvette, 467 µl of ethanol and 500 µl of DPPH 60 µM solution in ethanol were added. Samples were incubated for 20 minutes in a room in the darkness at room temperature. Antioxidant activity was determined by decolourization of DPPH at 517 nm wavelength using UV spectrophotometer. Ascorbic acid is used as a positive control. Antioxidant activity is determined by percentage reduction of DPPH using equation:

\[
\text{Scavenging activity (\%)} = \frac{A_{\text{DPPH}}(t) - A_{\text{sampel}}(t)}{A_{\text{DPPH}}(t)} \times 100
\]
Where A DPPH is the absorbance of DPPH and A sample is the absorbance of sample extract. All tests were run in triplicates (n=3) and average values were calculated.

2.3.2. Superoxide anion radical scavenging activity. The measurement of the superoxide anion radical scavenging activity of plant extracts was carried out according to the method as reported by Liu et al. [7]. Superoxide radicals were formed on the PMS-NADH system with NADH oxidation and tested by NBT reduction. In this experiment, superoxide radicals were formed in 3 ml Tris HCl buffer containing NBT, NADH and extract solutions. The reaction was initiated by addition of the PMS solution into the mixture. The mixture was subsequently incubated at 25 °C for 5 min and the absorption on the spectrophotometer was measured at 560 nm. Decreased absorption values indicate an increase in superoxide anion radical scavenging activity. Percentage of activity was calculated based on the ratio of absorption value on the extract sample to the absorption of the control. Ascorbic acid was used as a positive control. The activity was presented in the form of IC50 value (inhibitory concentration, concentration causing 50% inhibition of radical formation) to that of IC50 value of ascorbic acid.

2.4. Enzyme Inhibitory Assay

2.4.1. α -Glucosidase inhibitory activity assay. α-Glucosidase inhibitory activity was evaluated according to the method by Kim et al. [8]. The test solution contained 150 µl of 0.1 M sodium phosphate buffer, 0.1 UN α-glucosidase and extract concentration (1.5 - 200 µg/ml). The test mixture was pre-incubated at 37°C for 10 min. Subsequently, 50 ml of 2 mM p-nitrophenyl α-D-glucopyranoside in 0.1 M sodium phosphate buffer was incubated at 37°C for 25 min. The reaction was stopped by adding 50 ml of 0.1M sodium carbonate (Na2CO3). The yellow color formed was measured by a spectrophotometer at a wavelength of 405 nm. Mixtures containing α-glucosidase enzymes but without extracts were used as controls with 100% enzyme activity, and quercetin was used as a positive control.

\[
\%\text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100
\]  
(2)

The inhibitory activity was presented in the form of IC50 (inhibitory concentration, concentration causing 50% inhibition of enzyme) to then be compared to the IC50 value of quercetin.

2.4.2. A -Amylase inhibitory activity assay. The test sample (1.5 – 200 µg/mL) in test tube was added to 500 µl 0.02 M sodium phosphate buffer (pH 6.9) containing 0.5 mg/ml of the α-amylase enzyme solution and pre-incubated at 25°C for 10 min. After pre-incubation, 500 µl 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each test tube. The subsequent mixture was incubated at 25°C for 10 min. The reaction was terminated by adding 1 ml of the 3.5-acid dinitrosalilsic dye reagent. The reaction tube was incubated in boiling water for 5 min and then cooled to room temperature. The reaction mixture was then added with 10 ml of distilled water and measured by UV spectrophotometer at 540 nm wavelength [9]. Quercetin which is an α-amylase inhibitor is used as a positive control.

2.5. Total phenolic content analysis

Total phenolic content analysis was carried out with reference to the modified method of Yangthong et al. [10]. The total phenol content analysis was performed using a UV spectrophotometer at room temperature (25°C) at 765 nm. A calibration curve was made using gallic acid. The sample was put into a reaction tube of 0.3 ml, with 1.5 ml of Folin-Ciocalteu reagent and 1.2 ml of Na2CO3. Gallic acid was used as a positive control. Samples were incubated for 60 minutes in a room in the darkness at room temperature. After 60 min incubation, absorbance of the test sample was measured by using a UV / VIS spectrophotometer at 720 nm. The total concentration of phenol was calculated by the
standard calibration curve calibration expressed in mg/g gallic acid equivalent (GAE). The total content of phenol was calculated using the formula:

$$ C = \frac{c \cdot v}{m} $$  

(3)

where C is the total phenolic content (mg GAE/g), c is extract concentration (µg/ml), v is volume and m is extract weight (µg).

2.6. Total flavonoid analysis

The method used refers to Sharma et al. [11] with modification, especially in terms of sample weight used. Total flavonoid test method was performed using UV spectrophotometer at room temperature (25°C) and with maximum absorbance at 500 nm. The standard catechin calibration curve was made with serial catechin concentration (0-10 µg/ml). The sample was put into a 0.1 ml reaction tube, then added with distilled water of 0.7 ml, 0.1 ml NaNO$_2$ (5%) solution, AlCl$_3$ (10%) solution 0.1 m and 0.5 ml of NaOH (1 M), after which the solution was incubated for 10 minutes in a room in the darkness at room temperature. After incubation for 10 min, the absorbance of the test sample was measured by using a UV spectrophotometer at a wavelength of 500 nm. The total flavonoid content was calculated by a standard catechin curve expressed in mg CE/g Catechin equivalent (CE). The total flavonoid content was calculated using the formula (3) above.

2.7. Total proanthocyanidin content analysis

The analysis of proantocyanidin in plant extracts was carried out with reference to Porter et al. [12]. Six ml of a n-butanol/HCl solution (950 ml of n-butanol and 50 ml of conc. HCl), 1 ml of extract solution, 0.2 ml of ferric ammonium sulfate in 2 mol/l HCl were added and mixed. The mixture vial was further heated in a water bath at a boiling temperature of 50 minutes. Then the vial was cooled and the solution was transferred to a volumetric flask and added with a solution of n-butanol/HCl up to 25 ml. The absorption was measured on a spectrophotometer at a wavelength of 550 nm. Pelargonidin was used as a standard. The results were calculated based on the absorption unit at 550 nm per 1 mg extract.

3. Results and Discussions

α-Glucosidase is one of the key enzymes that play a role in the absorption of carbohydrates in the body. On the other hand, α-amylase is an endoglucanases enzyme that hydrolyses the 1,4-glucosidic binders in the starch. These enzymes have been known to be therapeutic targets for controlling hyperglycaemia that may potentially lead to diabetes [13,14].

*E. zwageri* stem bark was extracted and evaluated its antidiabetic and radical scavenging activities. Maceration done with ethanol gave ethanolic extract 8.62% as presented in Table 1.

**Table 1.** Yield of *E. zwageri* Stem Bark Extract and Radical Scavenging Activities

| Samples     | Scientific name | Part used  | Extract yield (%) | DPPH IC$_{50}$ (µg/ml) | Superoxide IC$_{50}$ (µg/ml) |
|-------------|-----------------|------------|------------------|------------------------|-------------------------------|
| Ulin        | *E. zwageri*    | Stem bark  | 8.62             | 44.90                  | 30.47                         |
| Ascorbic acid|                 |            |                  | 3.10                   | 20.64                         |

The results on DPPH free radical scavenging activity assay showed that the *E. zwageri* extract scavenged the radicals with IC$_{50}$ 44.90 µg/ml. The value was much higher than the IC$_{50}$ of ascorbic acid, the reference antioxidant that showed 3.10 µg/ml. In superoxide radical scavenging assay, the plant extract showed better activity than against DPPH. The IC$_{50}$ of plant extract to scavenge the radicals was 30.47 µg/ml. In the assay, ascorbic acid showed the IC$_{50}$ 20.64 µg/ml. The assay of
radical scavenging activity showed that the *E. zwageri* stem bark extract was more active to scavenge the superoxide radicals rather than DPPH.

Furthermore, the inhibitory activity against α-glucosidase and α-amylase enzymes of the *E. zwageri* stem bark extract was presented in Table 2. The results showed that the extract inhibited the α-glucosidase activity with IC$_{50}$ 58.45 µg/ml. The value was higher than the IC$_{50}$ of the control, quercetin, showing 2.00 µg/ml. In α-amylase inhibitory assay, the extract showed IC$_{50}$ 9.04 µg/ml that was close to the IC$_{50}$ of quercetin, showing 4.04 µg/ml. In relation to a scientific validation of ethnopharmacological information of *E. zwageri* stem bark as natural antidiabetic agent, the results indicated that the activity of *E. zwageri* is more effective as α-amylase inhibitor rather than the α-glucosidase.

**Table 2. Antidiabetic Activity of *E. zwageri* Stem Bark Extract by Means of α-Glucosidase and α-Amylase Inhibitory Assays.**

| Samples | Scientific name | Part used | α-Glucosidase IC$_{50}$ (µg/ml) | α-Amylase IC$_{50}$ (µg/ml) |
|---------|----------------|-----------|-------------------------------|-----------------------------|
| Ulin    | *E. zwageri*    | Stem bark | 58.45                         | 9.04                        |
| Quercetin |                |           | 2.00                          | 4.04                        |

Results of total phenolic content, total flavonoid content and total proanthocyanidin content analyses from the stem bark extract of *E. zwageri* were shown in Table 3. The results showed that the extract contains 31.28 mg GAE/g extract, 30.48 mg CE/g extract, and 183.30 mg PE/g extract represented the total phenolic, total flavonoid and total proanthocyanidin contents, respectively. The results suggested the potential contribution of phenolic, flavonoid and proanthocyanidin compounds in scavenging and inhibition of the extracts to radicals and key enzyme of diabetes. Several studies have shown that there are differences in the ability of phenolic compounds to inhibit the activity of α-glucosidase and α-amylase enzymes. In relation with that, *Orthosiphon stamineus* and sugar molasses have been reported to possess a good inhibitory activity on α-amylase. Although they are also good inhibitors of the α-glucosidase enzyme, the sequence of activity against these two enzymes is different [15,16].

**Table 3. Total Phenolic, Total Flavonoid and Total Proanthocyanidin of *E. zwageri* Stem Bark**

| Samples | Scientific name | Part used | Total Phenolic (mg GAE/g extract) | Total Flavonoid (mg CE/g extract) | Total Proanthocyanidin (mg PE/g extract) |
|---------|----------------|-----------|----------------------------------|----------------------------------|----------------------------------------|
| Ulin    | *E. zwageri*    | Stem bark | 31.28                            | 30.48                            | 183.30                                 |

The stem bark of *E. zwageri* is used by the Kutai ethnic in Indonesia for treating the diabetes in traditional manner. To the best of our knowledge, reports on the biological active compounds from *E. zwageri* are very limited. Some neolignans have been isolated from the heartwood of the plant and reported to be the responsible compounds for the durability of the wood [17]. Furthermore some lignans have also been isolated from the plants as reported by Yoosu et al. [18]. However, no extensive reports on the biological activity and related biological active compounds have been reported from the stem bark of *E. zwageri*. Isolation and identification of the active compounds from the plant stem bark are required to answer the mechanism of the bioactivity. Regarding the sustainability of plant source, the efforts toward the bioproduction of the active compounds of *E. zwageri* should be put in more priority rather than the extensive uses of the plant materials as the main source.
Currently, efforts to find alternative sources for the treatment of type 2 diabetes are in increase, especially those from medicinal plants having high antidiabetic activity and with a lower risk of side effects than commercial drugs currently available. In line with this, the research has shown that natural material-based compounds and natural-based products have the potential to have excellent ability to inhibit the activity of key enzymes that cause diabetes and with low side effects [19-21]. Therefore, the sustainable uses of plant sources as natural antidiabetic offer good solution for diabetes treatment.

In addition, regarding the information where medicinal plants were collected, efforts to strengthen the benefit sharing aspect to the community where samples of plants are collected need to be continued.

4. Conclusions
An ethnobotanically-selected medicinal plant, *E. zwagery* has been investigated its antidiabetic and antioxidant activities. The results informed that the stem bark ethanolic extract of the plant showed good antidiabetic activity only for α-amylase inhibition and weak activity to α-glucosidase. The extract was more active to scavenge the superoxide radical than DPPH. Further investigation is needed to find the responsible compound in the plant and to explore possibility of bioproduction for the active compounds.

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