α-Glucosidase Inhibitory Activity from Ethyl Acetate Extract of Antidesma bunius (L.) Spreng Stem Bark Containing Triterpenoids

Marista Gilang Mauldina, Rani Sauriasari, Berna Elya

Department of Pharmacy, Faculty of Pharmacy, Universitas Indonesia, Depok, Indonesia

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ABSTRACT
Background: Buni (Antidesma bunius [L.] Spreng) has been used as a traditional antidiabetic agent in Asia. Objective: The mechanism of antidiabetic properties was studied in this study by determine its α-glucosidase inhibitory activity. Method: Inhibition of α-glucosidase was performed in all fraction of Buni stem bark with acarbose and miglitol as standards. The half maximal inhibitory concentration (IC₅₀) value of acarbose and miglitol was 5.75 and 59.76 μg/mL respectively while ethyl acetate (EtOAc) fraction was the most active fraction with IC₅₀ of 19.33 μg/mL. Three isolates (B1, B2, and B3) were found in the EtOAc fraction and elucidated by infrared, hydrogen-nuclear magnetic resonance, ¹³C-carbon-nuclear magnetic resonance, and two-dimensional nuclear magnetic resonance. Result: The chemical structures of the isolates were identified by the spectrums when compared with literature which concluded that B1 is friedelin, B2 is β-sitosterol, and B3 is betulinic acid. Inhibition of the α-glucosidase assay showed IC₅₀ values of B1, B2, and B3 were 19.51, 49.85, and 18.49 μg/mL, respectively. Key words: Antidesma bunius (L.) Spreng, triterpenoid, α-glucosidase inhibitory activity.

SUMMARY
• α-Glucosidase inhibitory activity assay was performed in n-hexane, ethyl acetate (EtOAc), methanol fraction of Buni (Antidesma bunius [L.] Spreng) stem bark and miglitol
• EtOAc fraction from the liquid chromatography has the highest inhibitory activity against α-glucosidase
• The chemical structures of the isolates were identified by the spectrums infrared, hydrogen-nuclear magnetic resonance, ¹³C-carbon-nuclear magnetic resonance, and two-dimensional nuclear magnetic resonance, then compared with literature which concluded that B1 is friedelin, B2 is β-sitosterol, and B3 is betulinic acid
• Betulinic acid and friedelin showed the highest α-glucosidase inhibitory activity.

INTRODUCTION
Euphorbiaceae family is one of the widespread plants in Indonesia that has beneficial health properties that potential to be studied. Some of the Euphorbiaceae was reported for having a hypoglycemic activity, for example, Phyllanthus emblica Linn, Phyllanthus sellowianus, Antidesma celebicum (Miq), and Antidesma bunius (L.) Spreng. Hypoglycemic activity is the main character for identifying the antidiabetic agent. Asian countries, including Indonesia, contribute to >60% of the world’s diabetic population as the prevalence of diabetes is increasing in this country. One of the widely used antidiabetic drugs is α-glucosidase inhibitor. A. bunius (L.) Spreng which commonly known as Buni, is an edible plant found in Asia, including Indonesia. Although some preliminary studies on the antidiabetic properties of this plant have been reported, the results were still conflicting. The previous study showed that the 80% EtOH extract of Buni stem bark strongly inhibited α-glucosidase with half maximal inhibitory concentration (IC₅₀) value of 3.90 μg/mL. Another research in contrary stated that ethyl acetate (EtOAc) fraction of Buni stem bark has the highest activity with IC₅₀ value of 5.73 μg/mL. Moreover, based on our knowledge, there is no information regarding the active chemical substance of Buni stem bark. Therefore, we designed this experiment to confirm the α-glucosidase inhibitory activity from ethyl acetate extract of Antidesma bunius (L.) Spreng stem bark containing triterpenoids.

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Abbreviations used: IC₅₀: Half maximal inhibitory concentration; H-NMR: Hydrogen-nuclear magnetic resonance; C-NMR: Carbon nuclear magnetic resonance; 2D-NMR: Two dimensional-nuclear magnetic resonance; EtOH: Ethanol; EtOAc: Ethyl acetate; MeOH: Methanol; CHCl₃: Chloroform; DMSO: Dimethyl sulfoxide; EtF: Ethyl acetate fraction; Na₂CO₃: Sodium carbonate; IR: Infrared; TGR5: Transmembrane G protein-coupled receptor 5; EC₅₀: Half maximal effective concentration

Correspondence:
Dr. Rani Sauriasari,
Faculty of Pharmacy, Universitas Indonesia,
Depok 16424, Indonesia.
E-mail: rani@farmasi.ui.ac.id
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present study to determine the highest α-glucosidase inhibitor activity fraction of Buni stem bark and identify its chemical substances.

MATERIALS AND METHODS

Plant material

Buni stem bark was collected and identified in The Center for Plant Conservation of Bogor Botanical Garden in January 2013 with the authentic number 2467/IPH.3.02/KS/VI/2013. The specimen was stored in Herbarium of Pharmacognosy, Natural Product Medicine Laboratory, Faculty of Pharmacy Universitas Indonesia.

Chemicals

α-Glucosidase enzyme was purchased from Sigma Chemical Co., p-nitrophenyl-α-D-glucopyranoside was purchased from Sigma Chemical Co., miglitol was purchased from Tokyo Chemical Industry, acarbose was purchased from Sigma Chemical Co., n-hexane, ethanol (EtOAc), methanol (MeOH), chloroform (CHC$_3$), DMSO, and dichloromethane.

Extraction

Air-dried ground stem bark of Buni (2.4 kg) was macerated 8 times, each with 10 L of 80% MeOH at room temperature for 72 h and then evaporated at 40°C using vacuum rotary evaporator.

Fractionation and isolation

The extract was dispersed in water with the ratio of 1:1 and then performed with liquid partition chromatography used n-hexane, EtOAc, and MeOH. Each fraction was evaporated at 40°C using vacuum rotary evaporator until being viscous. The evaporated fraction was then tested by α-glucosidase inhibitory activity in vitro assay. A portion of fraction (25.0 g) with the highest α-glucosidase inhibitory activity was subfractionated by column chromatography with silica gel (Φ2.0 cm × 40 cm) as stationary phase and eluted with the gradient mixture of n-hexane, EtOAc, and MeOH. The mobile phase was started from n-hexane-EtOAc (100:0), and then, the polarity was enhanced to n-hexane-EtOAc (0:100) until EtOAc-MeOH (0:100). Each fraction was combined based on their TLC spot and gave 11 fractions, namely: EtF I (F1–7), EtF II (F8–11), EtF III (F12–16), EtF IV (F17–18), EtF V (F19–29), EtF VI (F30–35), EtF VII (F36–47), EtF VIII (F48–80), EtF IX (F81–108), EtF X (F109–116), and EtF XI (F117–130).

α-glucosidase inhibitory activity assay

All fractions were tested for their ability in inhibiting α-glucosidase using in vitro assay. The procedure refers to Kim et al.[7] The sample made into 5 variant concentrations (from 5 to 100 µg/mL) in volumetric flask, and then 30 µL of each concentration was added with 36 µL phosphate buffer pH 6.8 and 17 µL p-nitrophenyl-α-D-glucopyranoside 5 mM. Furthermore, the mixture solution was incubated for 5 min at 37°C. To this solution, 17 µL of α-glucosidase 0.15 unit/mL was added after the first incubation, then incubated again for 15 min at 37°C. After the second incubation was finished, 100 µL of Na$_2$CO$_3$ 267 mM was added into the solution to stop the enzymatic reaction. Solution absorbance was measured with a microplate reader at λ 405 nm. The blank solution was tested by adding Na$_2$CO$_3$ right after the first incubation and α-glucosidase after the second incubation. Acarbose and miglitol were tested as standards. The inhibition percentage was calculated by following formula.

\[
\text{Blank absorbance} - \text{Sample absorbance} \times 100\%
\]

The IC$_{50}$ were defined as the concentration of extract that inhibits 50% of α-glucosidase activity.

Structure identification

The chemical structure of isolate was determined using infrared (IR), 1H-hydrogen-nuclear magnetic resonance (H-NMR), 13C-carbon-nuclear magnetic resonance (C-NMR), and two-dimensional nuclear magnetic resonance (2D-NMR) (JEOL, 500 MHz).

RESULTS AND DISCUSSION

α-Glucosidase inhibitory activity assay was performed in n-hexane, EtOAc, and MeOH fraction of Buni stem bark [Figure 1]. Table 1 shows that the ethyl acetate fraction from the liquid chromatography has the highest inhibitory activity against α-glucosidase with IC$_{50}$ value of 19.33 µg/mL while acarbose and miglitol as standards have IC$_{50}$ value of 5.75 and 59.76 µg/mL, respectively. It concluded that ethyl acetate fraction has a higher α-glucosidase inhibitory activity than miglitol, in line with the previous study.[6]

EtOAc fraction was subfractionated by column chromatography and gave 11 subfraction. EtF II (8–11) and EtF IV (F17–18) produce white needles isolate that soluble in CHCl$_3$ on recrystallization, named B1 (11.43 mg) and B2 (10 mg). Further purification was done by repeated column chromatography in EtF V (F19–29) and produced white powder isolate that soluble in DMSO, named B3 (10.22 mg). Each isolate was tested for the inhibition of α-glucosidase and gave B3 as the most active isolate with IC$_{50}$ value of 18.49 µg/mL as shown in Table 2. The isolated compounds were identified by spectroscopic analysis as well as by comparison of their spectral data with previously reported values.

IR spectrum of B1 exhibited carbonyl (vmax: 1716.7 cm$^{-1}$), methyl, methylene, and methine (vmax: 2920.4; 2868.6; 1462.09; 1388.79 cm$^{-1}$). The 1H-NMR spectrum of B1 using CHCl$_3$ as solvent

**Table 1:** Half maximal inhibitory concentration value of standards and Buni stem bark fraction

| Sample       | IC$_{50}$ (µg/mL) |
|--------------|-------------------|
| Miglitol     | 59.76             |
| n-hexane     | 27.42             |
| EtOAc        | 19.33             |
| Methanol     | 21.07             |

IC$_{50}$: Half maximal inhibitory concentration; EtOAc: Ethyl acetate

**Table 2:** Half maximal inhibitory concentration value of the isolates from subfractionated ethyl acetate fraction

| Isolate | IC$_{50}$ (µg/mL) |
|---------|-------------------|
| B1      | 19.51             |
| B2      | 49.85             |
| B3      | 18.49             |

IC$_{50}$: Half maximal inhibitory concentration
revealed signals for 8 methyl of triterpenoid (0.71(s); 0.86(m); 0.94(s); 0.99(d); 1.26(s); 1.04(s) ppm). The 13C-1H-NMR spectrum showed the existence of 30 carbons which included 7 quaternary carbons (28.35; 29.80; 37.60; 38.46; 39.86; 42.33; and 213.5 ppm), 4 methine groups (42.94; 53.26; 58.39; 39.63 ppm), and 8 methyl groups (7.01; 14.83; 18.13; 18.86; 20.44; 31.96; 32.26; and 35.77 ppm). DEPT 135° spectrum showed 11 methylene groups (18.41; 22.47; 30.10; 32.59; 32.93; 35.51; 35.79; 36.17; 39.42; 41.45; and 41.71 ppm). Table 3 shows that these data were identical to those reported friedelin (C15H24O3).[8] 2D-NMR spectrum also reveals that it suggests triterpenoid typical friedelin [Figure 2]. Friedelin is derived from lupane triterpene. The previous study showed that friedelin isolated from Ficus drupacea leaves which tested for α-glucosidase inhibitory activity had 20.1% inhibition value at 100 μM.[9] IR spectrum of B2 exhibited hydroxyl (vmax: 2900 cm−1), vinyl (1732 cm−1), methyl, and methylene (vmax: 1391.79 cm−1; 1462.09). The 13C-NMR spectrum showed the presence of 30 carbons which consists of 3 quaternary carbons (36.59 ppm; 42.10 ppm; and 140.89 ppm), 11 methylene groups (–CH2) which was detected by DEPT 135° spectrums (11.9; 12.04; 18.84; 19.08; 19.4; and 19.89 ppm). Carbons on 140.8 and 121.7 ppm indicated the presence of C = C, while carbons on 71.6 ppm indicated the presence of –OH. The 1H-NMR spectrum of B2 revealed signals for olefinic proton on δ H 5.29 (m, 1 H) that similar with oxymethine proton δ H 4.68 and 4.55 which is similar with vinyl hydrogen (H-29). Signals δ H 1.63; 0.92; 0.75; 0.86; and 1.2 are similar with methyl groups. Based on the spectral data [Table 5], the structure of B3 was assigned as betulinic acid.

Table 3: The nuclear magnetic resonance spectral of B1 and friedelin[9]

|     | δ1H-NMR | δ13C-NMR |
|-----|---------|----------|
| B1  | Friedelin |          |
| 1   | 1.95 (2H, m) | 1.95     |
| 2   | 0.86 (6H, m) | 0.86     |
| 3   | -         | -        |
| 4   | 2.20 (2H, m) | 2.20     |
| 5   | -         | -        |
| 6   | 1.73 (2H, m) | 1.73     |
| 7   | 1.22 (4H, m) | 1.30     |
| 8   | 1.38 (6H, m) | 1.38     |
| 9   | -         | -        |
| 10  | 1.47 (8H, m) | 1.53     |
| 11  | 1.47 (8H, m) | 1.45     |
| 12  | 1.22 (4H, m) | 1.32     |
| 13  | -         | -        |
| 14  | -         | -        |
| 15  | 1.47 (8H, m) | 1.47     |
| 16  | 1.27 (3H, s) | 1.58     |
| 17  | -         | -        |
| 18  | 1.47 (8H, m) | 1.56     |
| 19  | 1.47 (8H, m) | 1.37     |
| 20  | -         | -        |
| 21  | 1.47 (8H, m) | 1.50     |
| 22  | 1.47 (8H, m) | 1.50     |
| 23  | 0.86 (6H, m) | 0.88     |
| 24  | 0.71 (3H, s) | 0.72     |
| 25  | 0.86 (6H, m) | 0.88     |
| 26  | 1.04 (3H, s) | 1.05     |
| 27  | 0.99 (6H, d, J=3.25 MHz) | 1.00     |
| 28  | 1.26 (3H, s) | 1.20     |
| 29  | 0.94 (3H, s) | 0.98     |
| 30  | 0.99 (6H, d, J=3.25 MHz) | 0.98     |

IR-NMR: Hydrogen-nuclear magnetic resonance; C-NMR: Carbon-nuclear magnetic resonance.

**Figure 2:** Friedelin

**Figure 3:** β-sitosterol
acid (C_{30}H_{50}O_{13}) [Figure 4] further supported by the 2D-NMR spectrum and spectral data reported from the literature.[12]

Betulinic acid and its derivates have been reviewed as one of natural constituents that showed potent α-glucosidase inhibitory activity.[13] Betulinic acid is also derived from lupane triterpene. The previous research stated that betulinic acid isolated from Morus alba root had a potentiality as antidiabetic agent under in vivo assay on streptozotocin-induced diabetic mice.[14] Another isolation of betulinic acid was done in Dillenia indica leaves, then tested by in vitro assay to inhibit α-glucosidase and α-amylase activity. The results were 52.2% inhibition to α-glucosidase and 47.4% inhibition to α-amylase at 50 μg/mL concentration of betulinic acid.[15] Another research reported that the betulinic acid gave 1.42 μmol/L EC_{50} into TGR5 receptor. TGR5 is one of the insulin secretion mediator from β-cell pancreas.[16] Those previous reports supported our results on antidiabetic activity of β-sitosterol, friedelin, and betulinic acid isolated from Buni stem bark.

**CONCLUSION**

Ethyl acetate fraction of Buni stem bark has a higher α-glucosidase inhibitory activity than miglitol. This study also afforded three triterpenes: friedelin (B1), β-sitosterol (B2), and betulinic acid (B3) from the stem bark of A. bunius (L.) Spreng, in which betulinic acid (B3) showed the highest α-glucosidase inhibitory activity.

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Conflicts of interest

There are no conflicts of interest.

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