

The Antidiabetic Effectivity of Indonesian Plants Extracts via DPP-IV Inhibitory Mechanism

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ABSTRACT

Background: Nature provides a rich source of antidiabetic medicines. More than 1.200 plants have been reported to have anti-diabetic activities and more than half of the drugs on the market come from natural products. Therefore, research for developing new antidiabetic drugs from natural sources is necessary to properly evaluate various mechanism of action, efficacy and safety at toxicological, physiological, pharmacological and molecular levels before these drugs applied in treatment of diabetes. This study aims to examine and determine the highest activity of 10 ethanol extracts from Indonesian plants selected for *in vitro* inhibitory activity of dipeptidyl peptidase - IV (DPP-IV). **Materials and Methods:** This research studied DPP-IV inhibitory mechanism from 10 plant extract, namely *Caesalpinia sappan*, *Cinchona officinalis*, *Elephantopus scaber*, *Foeniculum vulgare*, *Morus nigra*, *Muntingia calabura*, *Phyllanthus niruri*, *Psidium guajava*, *Rheum palmatum* and *Vernonia amygdalina* using the spectrophotometric method and Sitagliptin as a positive control. The effectiveness of inhibition was assessed by percent inhibition. Tannin removal test was performed to exclude false-positive results. **Results:** The ethanolic extract of *Caesalpinia sappan* exhibited DPP-IV inhibitory activity greater than 80%, which was not markedly different from that of Sitagliptin at 85%. Brazilin, an active

compound from *Caesalpinia sappan* gave the inhibition at greater than 78%. Tannin removal test for *Caesalpinia sappan* gave the inhibition at 74.16%. The High Performance Liquid Chromatography (HPLC) analysis result displayed that brazilin content on *Caesalpinia sappan* extract was 91.94%. **Conclusion:** Based on these results, *Caesalpinia sappan* ethanol extract has a potential effect as a better antidiabetic agent than other extracts used in this study.

Key words: Antidiabetic, DPP-IV, *Caesalpinia sappan*, Inhibitory activity.

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DOI: 10.5530/jyp.2019.11.34

INTRODUCTION

Diabetes mellitus (DM) is the fastest growing metabolic disease all over the world, identified by approximately 285 million people who have been diagnosed with DM to date, with this number predicted to increase by the year 2030.^{1,2} There are some mechanisms of antidiabetic drugs and currently the category of antidiabetic drugs working on the incretin pathway is more desirable.³ Incretin consists of a Glucose-dependent Insulinotropic Polypeptide (GIP) and Glucagon-Like Peptide-1 (GLP-1). However, GLP-1 has a short half-life (<2min) caused by its fast cleavage by DPP-IV.⁴ Therefore, DPP-IV inhibitors are the newest class of antidiabetic drugs approved by the FDA.⁵

Several synthetic DPP-IV inhibitors are available in the market such as sitagliptin, vildagliptin, saxagliptin and alogliptin. These synthetic drugs are expensive in their long term use for the purposes of therapy. Moreover, the study has shown that prolonged usage of these drugs causes unacceptable adverse effects such as pancreatitis, angioedema and infective disorders.⁶ The development of antidiabetic agents including natural products, is necessary to obtain drugs that are safe and have more efficient activity.

Jamu as a traditional medicine has been used in Indonesia for some generations. There are a number of experimental studies on type 2 diabetes that have reported that some herbs in jamu ingredients have been used in type 2 diabetes therapy and these drugs have been approved in human and animal research.^{7,8} It is assumed that herbal medicine provides a vulnerable alternative to treatment of type 2 diabetes. The

Diabetic Committee of the World Health Organization has made the recommendation for further research on herbal medicine as a treatment for diabetes.⁹

Stilbenes are polyphenolic compounds known to reduce postprandial blood glucose concentration levels.¹⁰ Resveratrol, a stilbene derivative effectively contributes to the protection of β cells in diabetes and reduces insulin levels in animals.^{11,12} These data showed that herbs containing stilbene may be useful in preventing and treating diabetes.

The extracts which contain stilbene are *Caesalpinia sappan*, *Cinchona officinalis*, *Elephantopus scaber*, *Foeniculum vulgare*, *Morus nigra*, *Muntingia calabura*, *Phyllanthus niruri*, *Psidium guajava*, *Rheum palmatum* and *Vernonia amygdalina* and they have been adopted for antidiabetic therapy, with their pharmacological properties being widely explored to develop new DPP-IV inhibitors.¹³ Based on this background this study aimed to evaluate 10 ethanolic extracts of selected Indonesian plants for *in vitro* inhibitory activity of DPP-IV.

MATERIALS AND METHODS

Extracts and chemicals

The extracts of *Caesalpinia sappan*, *Cinchona officinalis*, *Elephantopus scaber*, *Foeniculum vulgare*, *Morus nigra*, *Muntingia calabura*, *Phyllanthus niruri*, *Psidium guajava*, *Rheum palmatum* and *Vernonia amygdalina* were collected from Kebun Raya Bogor. The standard of Brazilin

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(MP Biomedicals, France), DPP-IV Assay kit (Cayman Chemical, USA), ethanol (Merck, Germany) and aquadest. Reagents for HPLC analysis were analytical grade and purchased from a local distributor.

Extraction

The powder of the dried plants, 10 g were macerated with 100 mL of 80% ethanol. The extracts were evaporated with a rotary vacuum evaporator (Buchi, Switzerland).

DPP-IV activity assay

The DPP-IV-Glo™ Assay was used to measure dipeptidyl peptidase IV (DPP-IV) inhibitor activity and was determined using Glomax (Promega, USA), with an excitation wavelength of 350-360 nm and emission wavelength of 450-465 nm.¹⁴ The samples were reconstituted in 80% ethanol to a final concentration of 1000 ppm. The assay used 96-wells microplate and the reaction per well included. 10 µL of the test sample mixed with 30 µL of assay buffer solution and 10 µL of DPP-IV solution. Enzyme control was prepared using 80% ethanol instead of the sample. Inhibitor control (Sitagliptin) was prepared using an inhibitor solution in place of the sample. Subsequently, 50 µL of substrate H-Gly-Pro-AMC substrate was added, then the microplate was incubated at 37°C for 30 min. Each test sample was analyzed in triplicate. The details of pipetting can be seen in Table 1.

Calculation of percent inhibition

Percent inhibition was calculated using the following formula in Eq. 1:

$$\% \text{ Inhibition} = \left(\frac{\text{Initial activity} - \text{inhibitor}}{\text{Initial activity}} \right) \times 100$$

Tannin elimination

Gelatin solution of 1% was added to the test filtrate. The flask was shaken at 100 rpm for 10 min at 25°C. The supernatant was dried in a waterbath and vacuum oven, then dissolved with 80% ethanol to ensure the tannin effect of the extract on DPP-IV inhibitory activity.¹⁵

Determination of brazilin in the extract by high Performance liquid chromatography (HPLC) analysis

The ethanolic extract of Brazilin was determined by HPLC to calculate Brazilin content. The system on the isocratic mode, nucleosil 100 C₁₈ (150 mm x 4.6 mm, 5 µm) was used as a stationary phase with a flow rate of 1.0 mL / min. The solvent used for separation was 0.3% acetic acid: acetonitrile (85.5:14.5). Brazilin was used as a standard compound for quantitative analysis. The standard 1.000 ppm Brazilin stock solution was performed by dissolving in 80% ethanol. Standard stock solutions were diluted to six standard solutions with concentrations of 200, 175, 150, 125, 100 and 75 ppm. Detection was carried out at 280 nm with the injection volume was 20 µL and 5 min retention time.¹⁶

Table 1: Pipetting summary.

	Assay Buffer	DPP (IV)	Solvent	Inhibitor	Substrate Solution
100% Initial Activity	30 µL	10 µL	10 µL		50 µL
Background	40 µL		10 µL		50 µL
Sitagliptin (Positive control inhibitor)	30 µL	10 µL		10 µL	50 µL
Samples	30 µL	10 µL		10 µL	50 µL

Table 2: DPP-IV inhibition activity of the extract and tannin elimination test.

No	Samples (100 ppm)	Part of plants	Inhibition (%)
1.	Sitagliptin (positive control)		85.18%
2.	Brazilin standard		78.30%
3.	<i>Caesalpinia sappan</i>	Heartwood	84.25%
4.	<i>Cinchona officinalis</i>	Stem bark	62.95%
5.	<i>Elephantopus scaber</i>	Roots	48.17%
6.	<i>Foeniculum vulgare</i>	Seeds	46.15%
7.	<i>Muntingia calabura</i>	Leaves	74.12%
8.	<i>Morus nigra</i>	Stem bark	51.0%
9.	<i>Phyllanthus niuri</i>	Aerial parts	70.48%
10.	<i>Psidium guajava</i>	Leaves	66.11%
11.	<i>Rheum palmatum</i>	Root	72.67%
12.	<i>Vernonia amygdalina</i>	Leaves	50.20%
13.	<i>Caesalpinia sappan</i> (tannin removal)	Heartwood	74.16%
14.	<i>Cinchona officinalis</i> (tannin removal)	Stem bark	22.50%
15.	<i>Phyllanthus niruri</i> (tannin removal)	Aerial parts	27.20%
16.	<i>Psidium guajava</i> (tannin removal)	Leaves	29.50%

RESULTS

DPP-IV inhibitory activity assay

The test results of DPP-IV inhibitory activity presented with percent inhibition values. The calculation result of the percent inhibition of the extract against DPP-IV enzyme can be seen in Table 2. The highest inhibitory activity of samples was obtained from *Caesalpinia sappan* extracts with percent inhibition value 84.25%, while Sitagliptin as positive control resulted in inhibition of 85.18%. Standard Brazilin was tested and gave the percent inhibition value of more than 78.30%.

Tannin elimination

Tannin content on the extract was removed and investigated to exclude false-positive results. Percent inhibition of the *Caesalpinia sappan* (without tannin) was 74.16%, *Cinchona officinalis* (without tannin) was 22.50%, *Phyllanthus niruri* (without tannin) was 27.20%, *Psidium guajava* (without tannin) was 29.50%.

Determination of brazilin in the extract by HPLC analysis

The amount of Brazilin on *Caesalpinia sappan* extract was determined by the HPLC method. Ethanolic extract and the standard solution of Brazilin were analyzed at wavelength 280 nm. The HPLC chromatogram of *Caesalpinia sappan* extract and Brazilin standard was shown in Figure 1. The linear equation for Brazilin was $y = 1542.3x - 2986.1$ ($R^2=0.9981$). The test result showed that Brazilin content was 91.94%.

DISCUSSION

Cayman's DPP-IV inhibitor screening assay used fluorescence-based method for screening DPP-IV inhibitors. This assay used the fluorogenic substrate Gly-Pro-Aminomethylcoumarin (AMC) to determine DPP-IV activity. DPP-IV releases the free AMC group by cleavage of peptide bond, resulting in fluorescence that can be analyzed using an excitation wavelength and emission wavelength.¹⁴

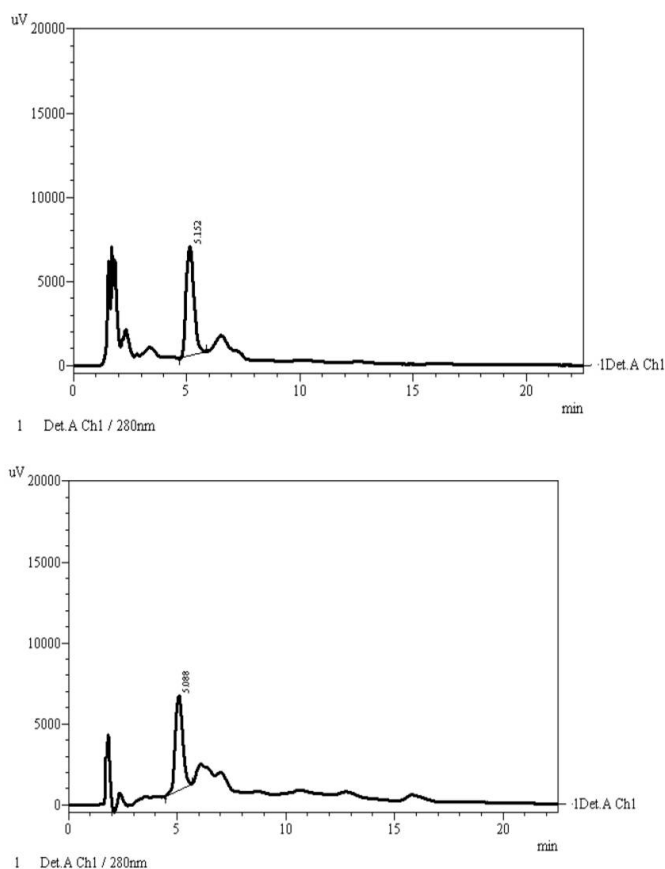


Figure 1: HPLC chromatogram of 100 ppm Brazilin standard (above) and 100 ppm extract (below).

DPP-IV inhibitory test demonstrated that *Caesalpinia sappan*, *Cinchona officinalis*, *Muntingia calabura*, *Phyllanthus niruri*, *Psidium guajava* and *Rheum palmatum* had inhibitory activity. The highest inhibitory activity of samples was obtained from *Caesalpinia sappan* extracts with a percent inhibition value of 84.25%. The percent inhibition values of *Caesalpinia sappan* were not much different than the percent inhibition of Sitagliptin as a positive control (85.18%). This shows that *Caesalpinia sappan* heartwood extract inhibits the activity of DPP-IV as well as Sitagliptin. The heartwood part of this plant contains brazilin. Brazilin standard solution was tested for its inhibitory activity against DPP-IV enzyme to strengthen the results of the *Caesalpinia sappan* extract inhibitory test. Brazilin standard solutions show significant inhibitory results of 78.30% on DPP-IV enzymes. These results prove that brazilin in *Caesalpinia sappan* extract has potential DPP-IV inhibitory activity. Some studies have suggested that the possible mechanism to explain inhibitory activity of brazilin in *Caesalpinia sappan* against DPP-IV enzyme is associated with insulin action. With the presence of insulin, brazilin can increase the rate of lipogenesis, glucose oxidation and regulate the enzymatic processes involved in glucose metabolism. Brazilin is associated with enhancement of insulin receptor function by a decrease in serin phosphorylation.¹⁷ Other studies have also reported that brazilin can stimulate glucose transport *in vitro* and decrease gluconeogenesis in hepatocytes isolated from diabetic rats. Brazilin is also reported to inhibit aldose reductase activity.¹⁸

Extract rich-in tannin, such as *Cinchona officinalis*, *Muntingia calabura*, *Phyllanthus niruri*, *Psidium guajava* and *Rheum palmatum* also showed potent inhibitory activity. The assay gave false-positive results if tannins

were present. Based on research, it has been known that tannins inhibit enzymes.¹⁷ Tannins give positive results in certain tests because of their reaction with enzyme proteins. Tannin is known to have specific properties to precipitate protein and non-specific inhibitory activity for some hydrolytic enzymes such as lipase, α -glucosidase, α -amylase and invertase.¹⁷⁻¹⁹ DPP-IV enzyme is a class of protein, that can react and bind to tannin and form precipitate, so the enzyme activity decreases as a result of enzyme complexation. A study by Adamczyk *et al.* found that tannin are more than just inhibitors, but rather modifiers of enzyme activity.²⁰ Removal of tannin and activity assay of the extract were conducted to prove DPP-IV inhibitory activity and to remove false-positive result caused by enzyme inhibition due the formation of tannin-substrate complexes on the extract. Percent inhibition of the *Caesalpinia sappan* extract (without tannin) was 74.16%, while the percent inhibition of other extracts was decreased significantly. These results indicate that tannins have a significant effect against lowering inhibition activity of *Cinchona officinalis*, *Muntingia calabura*, *Phyllanthus niruri*, *Psidium guajava* and *Rheum palmatum* and have no significant effect on the activity of DPP-IV enzyme inhibition of *Caesalpinia sappan* extract. The response of enzymes against tannins varied depending on the enzyme. The enzyme affinity varies with tannins and the influence of unknown tannins on a given enzyme is unpredictable.²¹ Our *in vitro* study demonstrated an appreciable inhibitory activity present in 10 samples.

HPLC analysis showed that brazilin content on *Caesalpinia sappan* extracts was 91.94%. High levels of brazilin in the extract showed that brazilin is a major compound in *Caesalpinia sappan* heartwood and this has been confirmed by other studies.²² Brazilin is a responsible compound for the activity of DPP-IV inhibition of *Caesalpinia sappan* extract and further research is necessary to determine the IC_{50} value and other possible activities of brazilin in *Caesalpinia sappan* extract.

CONCLUSION

Caesalpinia sappan, *Muntingia calabura*, *Rheum palmatum*, *Phyllanthus niruri*, *Psidium guajava* and *Cinchona officinalis* show the strong inhibition activity of DPP-IV. Based on the above-mentioned results, the ethanolic extract of *Caesalpinia sappan* has a potential effect as an antidiabetic agent.

ACKNOWLEDGEMENT

This research was supported by Directorate of Research and Community Engagement, Universitas Indonesia via Hibah PITTA 2018.

CONFLICT OF INTEREST

The Authors have no conflict of interest to declare.

ABBREVIATIONS

HPLC: High Performance Liquid Chromatography; **DPP-IV:** Dipeptidyl peptidase IV; **FDA:** Food and Drug Administration; **GLP-1:** Glucagon-Like Peptida-1; **Gly-Pro-AMC :** Glycyl-Pro-Aminomethylcoumarin.

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Article History: Submission Date : 12-09-2018 ; Revised Date : 24-12-2018; Acceptance Date : 25-01-2019.

Cite this article: Setyaningsih EP, Saputri FC, Mun'im A. The Antidiabetic Effectivity of Indonesian Plants Extracts via DPP-IV Inhibitory Mechanism. J Young Pharm. 2019;11(2):161-4.