

Difference of Acidic Adding Effect in Ethanol Extraction of White Mulberry Stem Bark (*Morus alba*) and DPP-4 Inhibiting Activity Screening for Identifying its Antidiabetic Potential

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History

- Submission Date: 15-04-2019;
- Review completed: 29-04-2019;
- Accepted Date: 09-05-2019.

DOI : 10.5530/pj.2019.11.126

Article Available online

<http://www.phcogj.com/v11/i4>

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ABSTRACT

Objective: Mulberry (*Morus alba*) is one of the plants that can be used to treat diabetes and bioactive compounds that play a role are apigenin. Apigenin compounds have been reported to have an antidiabetic effect and are found in the form of glycosides. To separate apigenin from its glycosides, it takes the process of hydrolysis using acid. This study aims to look at the differences between ethanol extracts without hydrolysis with acids and ethanol extracts which are hydrolyzed by acid and determine their activity as dipeptidyl peptidase-4 (DPP-4) inhibitors in vitro. **Methods:** *Morus alba* stem bark dry powder was extracted MAE using 96% ethanol with acid hydrolysis using HCl 2 N and extracted without acid hydrolysis then apigenin levels can be measured by each extraction process using HPLC. DPP-4 activity was evaluated using glycyl-prolyl-7-amino-4-methyl coumarin (Gly-Pro-AMC) substrate then the inhibitory effect of extracts was determined based on the number of free AMCs by measuring fluorescence at excitation wavelengths of 350-360 nm and emission wavelengths of 450-465 nm using micro-plate readers. Sitagliptin is used as a positive control of DPP-4 inhibition in this test. **Result:** The ethanol extraction method with acid hydrolysis can attract more apigenin compounds than the ethanol extraction method without acid hydrolysis. The level of apigenin in the sample of ethanol extract with acid hydrolysis was 0.16%, and in the ethanol extract without acid hydrolysis was 0.04%. The amount of inhibitory activity of DPP-4 *Morus alba* stem bark extract was 23%, which is 0.33 times the inhibition of sitagliptin activity. **Conclusion:** Extraction methods with acid hydrolysis are more effective in attracting apigenin compounds than without acid hydrolysis. *Morus alba* stem bark extract has an anti-diabetic effect through the mechanism of action of DPP-4 inhibitors can be used as a reference for therapy of diabetes mellitus from natural ingredients.

Key words: *Morus alba*, Apigenin, DPP IV.

INTRODUCTION

Dipeptidyl peptidase-4 (DPP-4) is an enzyme produced by various tissues in the body that works to deactivate the hormone incretin, namely GLP-1 and GIP when carrying out its functions in blood sugar homeostasis.¹ By inhibiting DPP-4 activity an effect will be obtained suppression of increased blood glucose so that the development of DPP-4 inhibiting agents is being carried out throughout the world as a therapy for diabetes mellitus.² Currently several anti-diabetic synthetic drugs with the DPP-4 inhibitor working mechanism have been marketed, such as sitagliptin, vildagliptin, saxagliptin, linagliptin and alogliptin.³ Although DPP-4 inhibiting agents have advantages such as not causing hypoglycemia and weight loss, and can be used as monotherapy or in combination with other anti-diabetic agents, these synthetic drugs have several side effects such as headache, diarrhea, joint pain, sore throat, nasal congestion and serious side effects, namely heart failure.³⁻⁵ So it is necessary to develop other sources that have DPP-4 inhibiting activities that are safer such as from natural ingredients.

Morus alba is biogeographically included in the family Moraceae and genus *Morus* that live in various countries including Indonesia.⁶ This plant is still used in traditional medicine to protect liver damage, strengthen joints, expedite urine expenditure, lower blood pressure, and reduce blood sugar levels.⁷ Research on leaves, root bark, and fruit *Morus alba* (MA) proves the existence of anti-diabetic effects.² However, there has been no study of MA stem bark as an anti-diabetic specifically as a dedication to DPP-4 activity.

The genus *Morus* has several species and phylogenetic relationships between them. Many studies have attempted to classify *Morus* species, resulting in varied numbers of designated *Morus* spp. The information from internal transcribed spacer (ITS) genetic sequences to the study of the members of the general accepted the genus *Morus*.

The classification of the ITS sequences of known interspecific hybrid clones into both the maternal and the paternal indicated that it was sufficient to distinguish interspecific hybrids in the genus *Morus*. Nanang Sasmita, *et al.* 2019's research states that the Adaptation of *Morus alba* and *Morus cathayana* plants in different climate and environment

Cite this article: Agusfina M, Saputri FC, Sakti AS, Mun'im A. Difference of Acidic Adding Effect in Ethanol Extraction of White Mulberry Stem Bark (*Morus alba*) and DPP-4 Inhibiting Activity Screening for Identifying its Antidiabetic Potential. *Pharmacogn J.* 2019;11(4):790-5.

conditions in Indonesia.⁸ Mulberry (*Morus alba* L. and *Morus cathayana* Hemsl.) Cultivation of business as silk feed in Indonesia is increasing, considering the benefits of mulberry economically, ecologically and pharmaceutically. The best mulberry species based on the whole parameter is *Morus alba* in West Java. This means that West Java's climate and environmental conditions are good for mulberry cultivation.

MA stem bark is known to contain apigenin, luteolin, quercetin, morine, caffeic acid, gallic acid, routine, umbelliferone, chlorogenic acid, and kaempferol.⁸ Apigenin is a compound of more than 20 compounds found in plants reported to have DPP-4 inhibitory activity.⁹ In a study by Junfeng Fan et al (2013) proving that apigenin compounds have potent DPP-4 inhibitory activity with IC₅₀ 0.14 ± 0.02 μM.²

Flavonoids in plants can be found in the form of glycosides in several glycosidic combinations.¹⁰⁻²⁹ Apigenin is one of the flavonoid compounds from plant secondary metabolites that are often found in the form of glycosides.²⁸ The hydrolysis method is used to release aglycones in the extract so that it can be further identified by HPLC.²⁹

MATERIALS AND METHODS

Samples

Stem bark of MA purchased from the local farmer on Sukabumi, West Java and determination by Lembaga Ilmu Pengetahuan Indonesia (LIPI).

Chemical

The standard of Apigenin was purchased from China (Shanghai Yuanye Bio-Technology Co.Ltd), Ethanol (Merck, Germany), DPP-4 Assay Kit (Cayman chemical, USA), Acetonitrile and methanol with analytical grade for HPLC was purchased from a local distributor.

Extraction process

The dried stem bark of MA was cleaned and ground to a powder using a plant disintegrator. Ethanolic extract : 10 gr sample powder was macerated with 100 ml distilled ethanol 96% on round bottom flask (Duran®, Germany) for 1 hour and then extraction by microwave assisted extraction (Modena MV-3002, with slight modification) with power 70 watt for 5 minutes.⁵ The extract were evaporated with a rotary vacuum evaporator (Rotavapor® R-215, Buchi, Switzerland) until obtained concentrated extract. The remaining solvent was removed using a vacuum oven at 37° C for 24 hours.¹⁰ Ethanolic extract with hydrolyzed acid: 30 mg dry ethanol extract added 3 ml of HCl 2 N then heated on boiling water for 10 minutes, then mixed with 3 ml of ethyl acetate, centrifuged for 20 minutes at 3,000 rpm. Ethyl acetate fraction was taken. The solvent residue was completely removed by drying at room temperature for 48 hours. The obtained extracts were stored in a refrigerator at -20°C until the analyzed.

Thin layer chromatography (TLC)

TLC used silica gel analytical plates test, 60 F₂₅₄ (Merck, Germany) as a stationary phase. Plate adjusted to size 4 x 6 cm. Sample solution

and standard solution (1000 μl/ml for both solutions) were spotted on the plate using a 100 μl capillary tube. The chamber saturated for 30 minutes using the mobile phase. Acetonitrile used as single mobile phase. Then the plate was put into the chamber which containing 5 ml mobile phase until the mobile phase reaches the elution limit. Then the plate dried immediately. After that the plate is read under UV light with a wavelength of 254 nm and 366 nm using UV cabinet (CAMAG, Switzerland).¹² The sample positive if the apigenin bind appears when reading the plate under UV light. Retention factor (Rf) is expressed as the movement of separating the extract. Rf values are calculated by dividing the distance traveled by the solvent.

High performance liquid chromatography (HPLC) analysis for determination of apigenin in the extract

The HPLC analysis was used C₁₈ reverse-phase column (Inertsil® ODS-3 5 μm, 250 x 4,6 mm, GL Science, Japan) and system (LC-20AT, Shimadzu, Kyoto, Japan), equipped with a UV-Vis detector (SPD-20A, Shimadzu, Japan) in the isocratic mode. Standard stock solution 1 mg/ml of apigenin in 80% ethanol and ethanolic extract with acid hydrolyzed used for this analysis. Sample was injected as much as 20 μl using a syringe (Agilent, Germany) then analyzed with detector wavelength 345 nm, flow rate of 0.5 ml. The mobile phase used acetonitrile-methanol with ratio 2:3. Apigenin in the extract was identified by comparing the retention time with the reference standard. Apigenin concentration was measured using the calibration curve of apigenin standard.

DPP-4 Inhibitory Activity Assay

DPP-4 activity is measured using The Glomax®-Multi Detection System (Promega, USA) with an excitation wavelength of 350-360 nm and emission wavelength of 450-465 nm. Analysis of each sample was carried out in a microplate containing 96 wells. The sample was dissolved with 80% ethanol to a final concentration of 1000 ppm. Enzyme control was prepared using 80% ethanol instead of the sample. Pipetting settings can be seen in the Table 1. After pipetting microplate incubated at 37°C for 30 minutes. Each test sample was analyzed in triplicate.¹³

Percent inhibition was calculated using the following formula in Equation 1:

$$\% \text{ Inhibition} = \left[\frac{\text{Initial Activity} - \text{Inhibitor}}{\text{Initial Activity}} \right] \times 100$$

RESULT

Thin layer chromatography (TLC) profiles

Thin Layer Chromatography Profile revealed that apigenin compounds were detected in ethanolic extract with hydrolyzed acid from MA stem bark with and spot colors as shown in Figure 1 when visualized under visible light, under UV light with a wavelength of 256 nm and 365 nm. While TLC from ethanolic extract without hydrolyzed acid did not show apigenin bands.

Table 1: Pipetting settings.

Well	Assay buffer	DPP-4	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

Determination of Apigenin in the extract by HPLC analysis

The amount of apigenin on *Morus alba* stem bark extract was determined by the HPLC method. Ethanolic extract with hydrolyzed acid and the standard solution of apigenin were analyzed at wavelength of 345 nm. The calibration curve for apigenin standard showed on the Figure 2. The linear equation was $y = 71248x - 1772,2$ ($R^2 = 0,99978$). Test result showed that apigenin content was 0.16%. The HPLC chromatogram of *Morus alba* stem bark extract and apigenin standard was shown in Figures 3 and 4.

DPP-4 inhibitory activity assay

Percent of inhibitory value is the result of DPP-4 inhibitory activity test. Table 2 shows the results of the DPP-4 inhibition test for Sitagliptin as a control inhibitor, Apigenin standard, and ethanolic with hydrolyzed acid extract. From these results it can be seen that

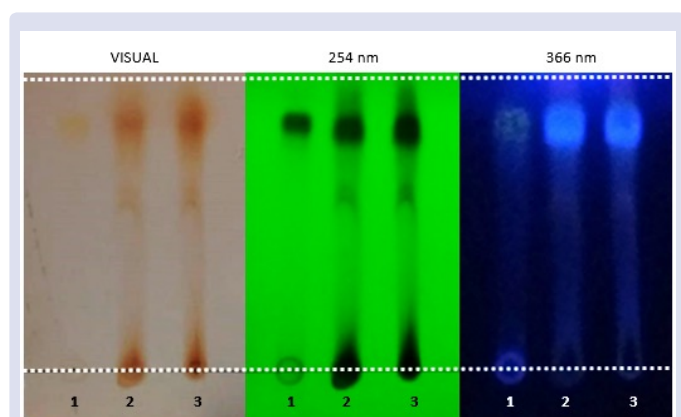


Figure 1: Apigenin bind on silica gel plate (1: apigenin standard, 2&3: ethanolic extract with hydrolyzed acid).

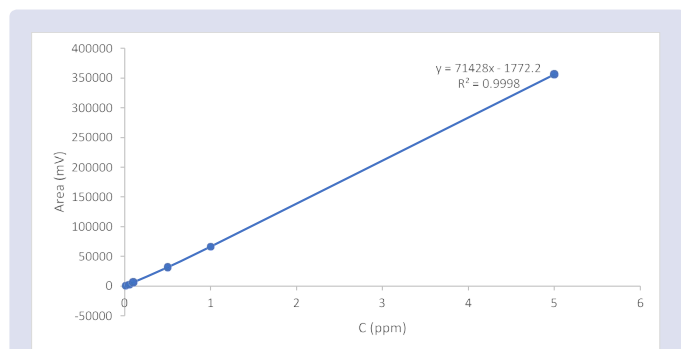


Figure 2: Apigenin calibration curve.

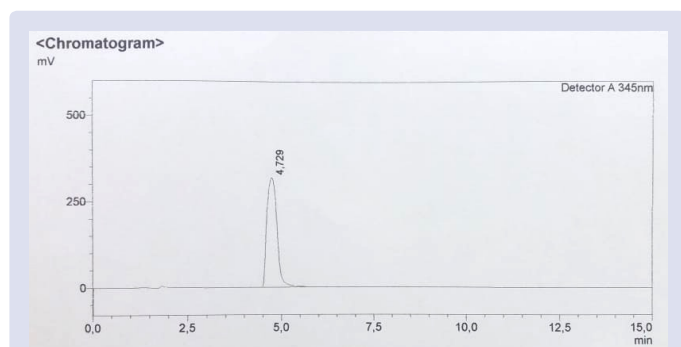


Figure 3: Chromatogram of standard apigenin.

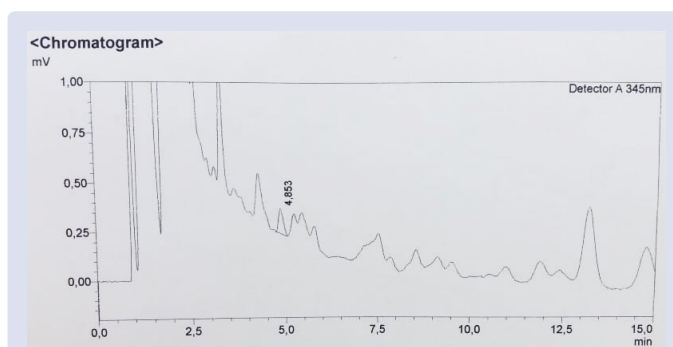


Figure 4: Chromatogram of Apigenin on the 100-ppm ethanol extract.

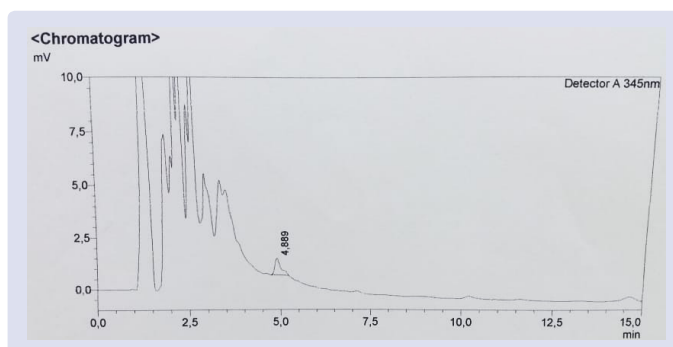


Figure 5: Chromatogram of apigenin on the 100-ppm ethanol extract with hydrolyzed acid.

Table 2: Result of DPP-4 inhibitory activity assay.

	% Inhibition
Sitagliptin	63.2%
Apigenin standard	42.13%
Ethanolic with hydrolyzed acid extract	23%

the large inhibition by apigenin standard is 0.67 times the inhibition of sitagliptin and the large inhibition by ethanolic with hydrolyzed acid extract is 0.33 times the inhibition of sitagliptin.

DISCUSSION

The genus of *Morus* has long been studied and has important uses in the pharmaceutical world. *Andalas* (*Morus macrourea* Miq.) Is one of the indigenous plants of Indonesia which has been designated as the mascot of West Sumatra. *Morus macrourea* Miq contains chemical compounds that can treat various diseases such as Betulinic Acid. In addition, this plant also contains chemical compounds that can inhibit the breeding of the HIV virus, antitumor melanoma in humans and prevent inflammation. In addition, andalasin A and andalasin B are isolated as the main components of *Morus macrourea* Miq which are potential compounds as antioxidant or tyrosinase inhibitors and can also be used as cosmetic ingredients for skin protection and bleaching or anti browning. Another member of the genus *Morus* is *Morus alba* L. and *Morus cathayana* Hemsl. Both of these plants are very potential as economic sources and basic ingredients for making pharmaceutical preparations. The main compound found in the genus *Morus* is apigenin.³¹

Apigenin is reported to have antioxidant effects that can be utilized in therapy for pancreatic β cell damage induced by oxidative stress.²² In other studies, apigenin is included in the group of flavonoid compounds which have reported anti-diabetic effects through DPP-4 inhibitory mechanisms.^{2,9}

In this study, Apigenin level from the ethanolic extract stem bark of MA with hydrolyzed acid compared to the ethanolic extract without acid hydrolysis. From TLC, apigenin band was performed on acid hydrolyzed MA ethanolic extract, while MA ethanolic extract which was not acid hydrolyzed showed no apigenin band. This is because apigenin which is a class of flavones often found in plants in the form of glycosides.²⁰ To be able to detect apigenin better, action is needed to break the bonds of the glycosides. Strong acids can help break the glycoside bonds so that this study uses concentrated HCl in the extraction process.²³

The selection of optimal separation conditions depends on the specificity of analyzed sample. Despite the huge progress in separation techniques, a complex matrix cannot be injected directly into the analytical instrument. Sample pretreatment is required to remove the matrix components which could interfere with an analyte and deteriorate quality of separation or detection. Sample pretreatment is frequently used to preconcentrate the analytes of interest from the target matrices. Some types of samples such as water and other fluids are suitable for relatively simple collection and preparation. Solid samples, including fruits, plants, or vegetables, require physical homogenization and more sophisticated pretreatment. Several methods have been developed for the determination of flavonoids in plant samples. In the next step hydrolysis was aimed at breaking the bonds between β -glycoses and sugar molecules. This bond commonly known that solid tissues must be shredded and homogenized before the analysis by liquid phase separation techniques. Sample preparation method included homogenization, liquid extraction, and acid hydrolysis.³³

Glycosides are organic compounds in the plant world. Glycosides consist of two parts, namely sugar molecules (saccharides) and aglycones. Sugar groups can bind aglycone groups in various ways. The most common bond is made by oxygen atoms (O-glycoses), sulfur (S-glycosides), Nitrogen (N-glycosides) or carbon (C-glycosides). Glycosides are generally solids which are soluble in water and alcohol but are slightly soluble in ether.

Glycosidic bonds are resistant to hydrolysis by alkalis but are easily broken by enzymatic reactions of dilute glycosides or mineral acids. The time needed to break the sugar bonds of flavonoids O-glycosides by acid hydrolysis is determined not only by the strength of the acid, but also by the nature of the bonding of sugar and where the sugar is attached to the nucleus of flavonoids (7-O-glycosides O-glycosides > 3-O-glycosides).³⁴

In this study, apigenin levels in MA ethanolic extract without acid hydrolysis was 0.04% and apigenin levels in MA ethanolic extract with acid hydrolysis was 0.16%. From these results it is known that the extraction method using acid hydrolysis attracts more apigenin compounds because the acid breaks the glycoside bond. However, the number of these compounds can be less or more with different extraction processes either from solvents or extraction methods.¹¹ So to get more apigenin amounts, further research is needed regarding solvents or better extraction methods.

Morus alba is one of the plants known to have anti-diabetic effects originating from traditional medicine and has been proven from various research results. Previous research found that moranoline compounds from MA root bark which have anti-diabetic activity.¹⁴ MA branches have also been tested for their anti-diabetic effects on STZ-induced mice where the results of this study indicate an increase in metabolic function in the pancreatic tissue resulting in a decrease in blood sugar level,²⁴ The MA fruit reported potential in the treatment of type 2 diabetes mellitus in studies using mice that had also been induced STZ,²⁵ and MA leaves were also reported to have anti-diabetic effects on STZ-induced mice by stimulating insulin secretion.²⁶ Meanwhile, the portion of the MA stem bark known to contain compounds

apigenin, one of the compounds that has a potent DPP-4 inhibitory activity,^{2,8,9} has not been studied for its anti-diabetic effect. So, this study examines the anti-diabetic effects of MA stem bark specifically through the mechanism of DPP-4 inhibitory action which is expected to complement the scientific literature on MA plants specifically related to the benefits of the treatment of diabetes mellitus.

DPP-4 inhibitor activity screening test uses Cayman's Kit based on the fluorescence method. This test analyzes the free fluorogenic Gly-Pro-Aminomethylcoumarin (AMC) substrate released by DPP-4 by removing peptide bonds to produce fluorescence that can be analyzed using excitation wavelengths and emission wavelengths using the Glomax[®]-Multi Detection System (Promega, USA).¹³ From this study it was known that the inhibitory activity of DPP-4 from extracts of the stem bark of the MA was 23%. When compared with the DPP-4 inhibitor control, namely sitagliptin, the inhibitory activity of DPP-4 by MA stem bark extract was 0.33 times the inhibitory of the DPP-4 produced by sitagliptin. While the large activity of DPP-4 inhibitors of standard Apigenin is 42.13%, which is 0.67 times the inhibitory activity of sitagliptin. From these results it is known that the MA stem bark has an anti-diabetic effect through the working mechanism of the DPP-4 inhibitor which can be used as a reference for diabetes mellitus therapy. DPP-4 inhibiting agent works by increasing incretin hormone (GLP-1 and GIP) which inhibits the release of glucagon so that it can increase insulin secretion and reduce blood glucose.²⁷

CONCLUSION

Thin Layer Chromatography Profile detected the presence of apigenin in ethanolic extract with hydrolyzed acid from MA stem bark with Rf 0.826 and the use of HPLC in the extract showed apigenin levels of 0.16% for ethanolic extract with hydrolyzed acid and 0.04% for ethanolic extract without hydrolyzed acid. It is mean hydrolyzed acid methods more effective than without hydrolyzed acid for extract apigenin.

In this study it was known that the inhibitory activity of DPP-4 from the extract of the MA stem bark was 23%. Compared to sitagliptin, the inhibitory activity of DPP-4 by extract of the MA stem bark was 0.33 times the inhibitory of DPP-4 by sitagliptin. While the large DPP-4 inhibitors activity of standard apigenin is 42.13%, which is 0.67 times the inhibitory activity of sitagliptin. From these results it is known that the MA bark has an anti-diabetic effect through the working mechanism of the DPP-4 inhibitor which can be used as a therapy for diabetes mellitus from natural ingredients.

ACKNOWLEDGEMENT

This study is supported by the PITTA 2018 grant from the Directorate of Research and Humanitarian Involvement (DRPM), University of Indonesia.

CONFLICTS OF INTEREST

The author states that in this study they did not have a conflicts of interest.

ABBREVIATIONS

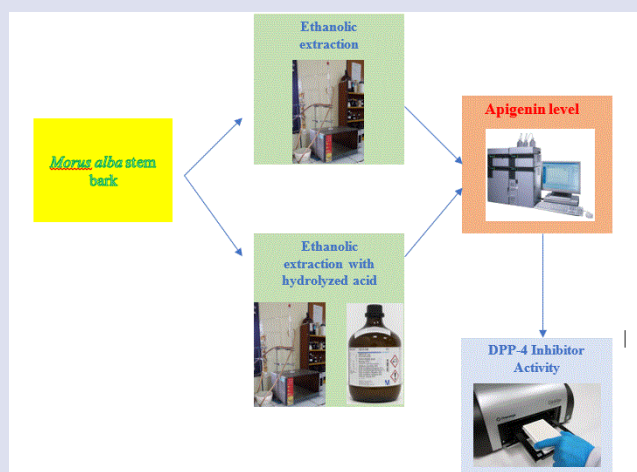
DPP-4: Dipeptidyl peptidase 4; HPLC: High Performance Liquid Chromatograph.

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GRAPHICAL ABSTRACT



SUMMARY

Extraction methods with acid hydrolysis are more effective in attracting apigenin (0.16%) than without acid hydrolysis (0,04%). *Morus alba* stem bark extract has an anti-diabetic effect through the mechanism of action of DPP-4 inhibitors (23%) can be used as a reference for therapy of diabetes mellitus from natural ingredients.

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Dr. Fadlina Chany Sapurti, M.Si., Apt.: Associate Professor at Department Pharmacology-Toxicology, Faculty of Pharmacy, Universitas Indonesia (UI) Depok, West Java, Indonesia. Her research focused on herbal plants and its activity on degenerative disease (such as diabetes mellitus, antihypertension and other cardiovascular disease).



Aditya Sindu Sakti, S.Farm., Apt.: Master student at Herbal Medicine, Faculty of Pharmacy, Universitas Indonesia. His focus research on natural products extraction, phytochemical analysis, dipeptidyl peptidase-4 inhibitory assays, and microbiological assays.

Cite this article: Agusfina M, Saputri FC, Sakti AS, Mun'im A. Difference of Acidic Adding Effect in Ethanol Extraction of White Mulberry Stem Bark (*Morus alba*) and DPP-4 Inhibiting Activity Screening for Identifying its Antidiabetic Potential. *Pharmacog J.* 2019;11(4):790-5.