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Phytochemistry and Antidiabetic Evaluation *in-vitro* and *in-vivo* of Ethyl Acetate Fractions from Sungkai Leaves (*Peronema canescens* Jack)

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ABSTRACT

The paper reports the *in vivo* and *in vitro* antidiabetic activity of bioactive compounds composed of ethyl acetate fractions from Sungkai (*Peronema canescens* Jack). The fractions are obtained from vacuum liquid chromatography (F1, F2 and F3). Phytochemicals screening, UV-Vis and FTIR spectrometry analysis is applied to detect secondary metabolite composed of the fractions. In addition, in vitro antidiabetic capability is measured inhibiting α-amylase activity, and in vivo evaluation is conducted using diabetic rat's model induced by alloxan. It was found that fraction collected as a white precipitate. The phytochemical analysis indicated positive for flavonoid. UV-Vis and FTIR spectra support the data. Moreover, capability of the fractions to inhibit α-glucosidase activity in the range 5-100 ppm does not show the activity. Conversely, the in vivo evaluation from fraction 2 at concentration 600 mg/Kg of body weight has antidiabetic in 50.16%. This result comparable to that of glibenclamide as a control. This finding lead to further evaluation for structural elucidation.

Key word: antidiabetic, *Peronema-canescens*, secondary-metabolite, flavonoid

INTRODUCTION

Diabetes mellitus is a degenerative condition marked by high blood glucose levels brought on by issues with insulin secretion in the body. If blood sugar levels are not controlled, it will cause short-term and long-term complications in patients. It can even lead to death [1]. International Diabetes Federation (IDF) 2019 reported that Indonesia was included in the ten countries with the highest number of people with diabetes, which was ranked seventh in the world after China, India, the United States, Pakistan, Brazil, and Mexico with around 10.7 million people with diabetes [2].

One alternative in dealing with diabetes mellitus is traditional treatment. This is in line with the World Health Organization (WHO) which recommends traditional medicine. Back to nature by utilizing the potential of natural ingredients, which has several advantages compared to the use of synthetic drugs, traditional medicine uses plants, microbes, and other sources [3]. The chemical composition of plants, particularly their biologically active constituents, is intimately tied to their function as medicines. Secondary metabolites such as flavonoids, alkaloids, saponins, tannins, and others are frequently discovered as bioactive substances in plants. among the plants that have potential as an antidiabetic medicinal plant is a Sungkai leaf (*P. canescens* Jack).

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Sungkai (*P. canescens* Jack) is one of the ethnobotanical plants used as a source of traditional medicine. It is endemic to Indonesia, and commonly found in Sumatra and Kalimantan Island [3]. The content of bioactive compounds in Sungkai leaves has been known to have antimicrobial [4], antiplasmodial [5], antioxidant [6], antihyperuricemic [7], anti-inflammatory [3], and antidiabetic activity. It was generally derived from ethanolic extracts to reduce blood glucose levels [8]. In addition, the diverse pharmacological activity of Sungkai correlate to the diverse bioactive components therein. Previously, phytochemical screening of the ethyl acetate extract from the leaves part contained some secondary metabolites such as alkaloids, flavonoids, tannins, and phenolic compounds [9]. Similar result is reported by Suryani et al (2024) [10] and Pratiwi et al (2023) [11]. This paper is disclosed our recent investigation toward their antidiabetic activity in vitro and in vivo of the fractions derived from the ethyl acetate fraction.

EXPERIMENT

Chemicals and instrumentation

The chemical used as abought from manufacturer include n-hexane, ethyl acetate, methanol (Sigma), Wagner's reagent (Merck), Meyer's reagent (Merck), Dragendorff's reagent (Merck), hydrochloric acid, silica gel for column chromatography, Mg powder, sulfuric acid, distilled water, iron(III) chloride, anhydrous acetic acid, alloxan, commercial glibenclamide, p-nitrophenyl-α-D-glucopyranoside (PNPG), sodium carboxymethyl cellulose (Na-CMC), dimethyl sulfoxide (DMSO), α-glucosidase enzyme and phosphate buffer solution.

The equipment used such as maceration bottles, rotary evaporator, column chromatography tube (Pyrex), analytical balance, TLC plate, injection syringe (One Med), 5 ml injection syringe (One Med), 3 ml injection syringe (One Med), animal scale, test animal cage, glucometer, 96 well microplate, microplate reader, UV-Vis spectrophotometer, FTIR spectrophotometer and ELISA kit.

Experimental procedure

Sample collection and preparation

The sample of Sungkai leaf (*P. canescens* Jack) is collected from locals in Koto Baru Hiang Village, Kerinci Regency, Jambi Province. The specimen is stored and deposited in University of Jambi. Then, samples were cleaned and dried at room temperature. It was further pulverized to form a powder.

Extraction procedure

A kilogram of sample powder was macerated in two repetitions. First maceration was conducted using n-hexane. It was left for over 2 x 24 hours. The n-hexane extract was separated by filtration. The sample residue was further macerated using ethyl acetate as solvent. It left for over 2x 24 hours and undergo repetition maceration. After filtration, the ethyl acetate extract was evaporated under reduced pressure to yield ethyl acetate fraction. In the meantime, the remaining residue was further macerated using methanol to afford methanol fractions.

Separation in TLC

Monitoring the separation of the fractions is conducted using thin layer chromatography (TLC). Silica based TLC F_{254} is used with dimension 1x5 cm. The eluent is chosen that give good spot separation. Staining reagent and UV lamp is used to detect the separated compounds.

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Separation with vacuum liquid chromatography.

The column of chromatography is filled with slurry of silica gel. It was left for several hours avoid column crack. Then, sample of ethyl acetate fraction was loaded onto the top of silica. It was further eluted using gradient polarity of solvent (n-hexane, ethyl acetate and methanol). Vacuuming was applied to separate the fractions, by collecting in the vials. After all fraction was eluted and collected, then, separation was stopped. The separated fractions were collected based on the similarity spots of retardation factor (Rf) in TLC. Vials with similar spots of Rf was collected as the same fractions.

UV-Vis spectrophotometry analysis

A 2 mL of the sample was put into a cuvette. The spectrum was observed at a wavelength of 200-800 nm to determine the absorbance value of the active compound at the maximum wavelength.

FTIR spectrophotometry analysis

A 0.2 g of KBr pellet was added with a drop of sample. Then, it was dried and identified using an FTIR spectrophotometer at a wave number of 4000-400 cm^{-1}.

Phytochemical screening

Analysis the presence of secondary metabolite composed in the fractions separated are undertaken following phytochemical test from literature [12].

Alkaloid test

It is required to dissolve the test sample in a few drops of 2N sulfuric acid. Three different ingredients, Dragendorff's Meyer's reagent, and Wagner's reagent were applied to the sample to examine. Meyer's reagent yields a yellowish white precipitate in the sample. Wagner's yields a brown precipitate. Dragendorff's yields a reddish-orange precipitate.

Flavonoid test

A few drops of strong HCl and Mg powder are applied to the sample to be evaluated. If the solution's color changes to orange and foam formed, the test is considered successful.

Saponin test

Saponin test can be done by foam test in hot water. The test results were positive if the bullae produced were stable for 30 minutes. It did not disappear when a drop of 2N HCl was added.

Phenolic test

Phenolic test was carried out by adding $FeCl_3$ and homogenized. If a blackish purple color is formed in the mixture, it indicates that there are phenolic compounds in the sample being tested.

Triterpenoid/Steroid test

The steroid test was carried out with the addition of concentrated sulfuric acid and anhydrous acetic acid as Liebermann-Burchard reagents in the sample which showed the formation of a purple or red color and then changed to blue purple or blue green indicating that there were triterpenoid/steroid compounds in the sample.

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Antidiabetic activity test *in-vivo*

The procedure to carried out using animal as a model following ethical standard by university, and the evidence of animal health certificate recorded as TN.01.03/176/DPKP /SKKH/2021. Groups of white mice were used with criteria of weight 20-30g, 2-3 months old, in healthy and normal condition.

Testing of antidiabetic activity begins with the acclimatization process of mice to the test environment for 1 week. Furthermore, diabetes induction was carried out, first measuring the blood sugar levels of mice (h_0). Alloxan was induced in mice with a dose of 150 mg/kgBW mice in all treatment groups (Table 1). After induction, mice were given food and drink. The condition of the mice was observed on the fourth day after induction (h_4) and the mice's blood sugar levels were measured. Diabetic mice with blood glucose levels >136 mg/dl were used as test animals. Before taking blood, mice must first have fasted for 8 to 12 hours while still being given drinking water. The next step was for mice to be given a test solution (suspension fraction and glibenclamide) which was adjusted to the group and the respective dose which lasted for 10 days. On the 10th day after administration of the test solution (h_{14}), the blood glucose levels of the mice were returned. Measurement of blood sugar levels in mice is done by taking blood from a vein that can be found at the tail's end of the mouse and then measuring blood sugar levels using a glucometer [13]. In addition to blood sugar levels, another variable observed in the test changes in body weight. The trial was divided into five treatment groups with the group divisions listed in Table 1.

Table 1. Group of tested animal with different treatment

Groups	Treatment
Negative control (K-)	Alloxan 150 mg/kgBW and Na-CMC 0.5%
Positive control (K+)	Alloxan dose 150 mg/kgBW and glibenclamide 3 mg/kgBW
F1	Alloxan dose 150 mg/kgBW and sungkai leaf fraction 150 mg/kgBW
F2	Alloxan dose 150 mg/kgBW and sungkai leaf extract 300 mg/kgBW
F3	Alloxan dose 150 mg/kgBW and sungkai leaf extract 600 mg/kgBW
Isolate	Alloxan dose 150 mg/kgBW and isolate 3 mg/kgBW

Note: F1, F2, F3 are sample prepared from fraction F2 isolated from VLC process of ethyl acetate fraction with different variation concentration as mentioned. K- and K+ is control sample negative and positive with composition as mentioned.

Antidiabetic activity test *in-vitro*

Procedure to evaluate *in vitro* antidiabetic evaluation using the α-glucosidase inhibition method following reference previously reported [14]. The test sample was divided into 60 μL, 50 μL of 0.1 M phosphate buffer pH 6.8, and 20 minutes of incubation at 37 C in a 96-well microplate. The test sample was replaced with 60 μL of a phosphate buffer solution to provide the blanks. Before incubation, p-nitrophenyl-α-D-glucopyranose (PNPG) 50 μL as much as 5 mM was put into a microplate and then incubated for 20 minutes at 37ºC. As a final step, the reaction was stopped by adding 160 μL of 0.2 M Na_2CO_3 solution into the well and the adsorbent was read at a wavelength of 425 nm with a microplate reader.

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RESULT AND DISCUSSION

Sample preparation and extraction

The Sungkai leaves picked from the plants are sorted in wet, and then washed to remove dirt attached. Then, it was dried at room temperature and placed in an open space and avoid direct exposed to sunlight. The drying process is carried out to prevent the growth of bacteria and fungi so that the sample is not easily damaged. After drying, the Sungkai leaf samples were then grounded into powder. This aims to reduce the particle size of the sample to enlarge the surface area. It eases the contact between the sample and solvent, to fasten the extraction process [15]. From a 4.0 kg of wet sample, a drying process reduced to 1.0 kg of powder. Maceration started using n-hexane. The nonpolar secondary metabolite is extracted with this solvent, and the residue is extracted further using ethyl acetate. This solvent separates the semi-polar secondary metabolite and left the polar compounds in the residue or in the methanol solvent [16]. This gradient strategy partition via extraction-maceration has beneficial toward the unstable secondary metabolite. Room temperature maceration does not give any issues on structure instability [17]. Maceration of the sample *P. canescens* Jack with ethyl acetate provide a brown-black saturated liquid. It is predicted semi polar secondary metabolite composed in the fraction. Similar extraction process of *Marsdenia tenacissima* leaves using ethyl acetate as solvent provided semi polar compound such as alkaloid, flavonoid, phenol, and saponin [18].

Phytochemical screening

Phytochemical screening was carried out to identify the bioactive compounds in the sample. Results of the partition-extraction of *P. canescens* Jack using n-hexane, ethyl acetate and methanol is tabulated in Table 2. The ethyl acetate extract-fraction give positive result for flavonoid, phenolic/tannin and triterpenoid steroid. Meanwhile, methanol extract fraction instead positive for triterpenoid saponin, and n-hexane extract provide a positive alkaloid. Similar finding is reported by Ku Halim et al. [19] using *P. cenescens* sample collected from Terengganu. Mostly phenolic and steroid compound was isolated. Further purification is led to isolate this compound.

Table 2. Phytochemical screening of the extract from *P. canescens* Jack

Secondary Metabolites	**Extract results from**		
	n-Hexane	Ethyl acetate	Methanol
Alkaloid	+	-	-
Flavonoid	-	+	+
Phenolic / Tannin	-	+	+
Triterpenoid saponin	-	-	+
Triterpenoid steroid	-	+	-
Terpenoid	-	-	-

Separation and purification

Purification further on ethyl acetate fraction use VLC, and silica gel applied as stationary phase. The gradient elution procedure is followed using n-hexane, ethyl acetate and methanol as solvent. It provides 40 vials and collected based on TLC into 3 fractions. Fraction F1 (vials 1-8), fraction F2 (vials 9-12) and fraction F3 (vials 13- 40). Secondary metabolite screening test was conducted for the fractions (Table 3). Phenolic / tannin and steroid is detected in F1. While F2 and F3 give positive result for flavonoid and steroid. However, it was

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collected a white precipitate in F2 (vial 9). It has a single spot result in TLC using various solvent that indicate purity. Further tests in conducted to indicate a positive for flavonoid secondary metabolite composed in F2.

Table 3. Phytochemical screening of fractions from ethyl acetate fraction

Secondary Metabolites	Collected fractions from ethyl acetate fractions		
	F1	F2	F3
Alkaloids	-	-	-
Flavonoids	-	+	+
Phenolic/Tanins	+	-	-
Saponins	-	-	-
Steroids	+	+	+
Terpenoids	-	-	-

UV-Vis and FTIR analysis

Analysis toward the fraction F2 using UV-Vis and FTIR spectrophotometry is displayed in Figure 1 and 2. Spectrum UV-Vis report the absorption band of compounds from 200-800 nm. It indicates electronic transition of molecule such as from $\pi \rightarrow \pi^*$ in aromatic ring or alkenes and $n \rightarrow \pi^*$ in aldehyde, ketone, and carboxylic derivates compound. It is observed that spectrum (Figure 1) has electronic transition for $\pi \rightarrow \pi^*$ (at 269 and 350 nm) and $n \rightarrow \pi^*$ (at 430 nm). This region characteristic aromatic band I (310-350 nm) and band II (255-280 nm) [20].

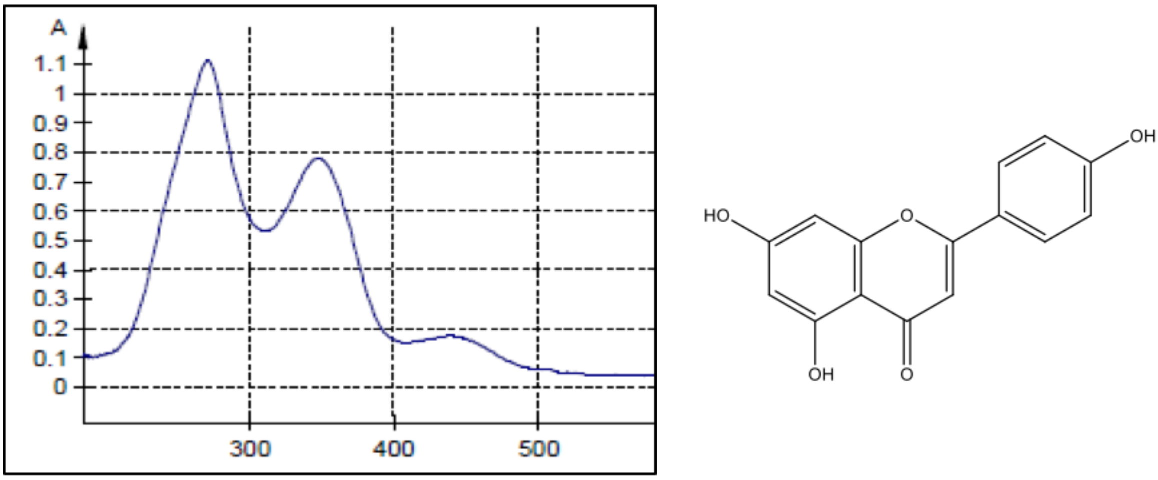

Figure 1. UV-Vis spectrum F2, and structure of flavonoid (4',5,7-trihydroxyflavone)

The spectra FTIR (Figure 2) correlate to the composition of functional groups contained in molecule of secondary metabolites of F2. The spectra band specific due to vibration of the functionality occurring. It is found hydroxyl group (-OH) attached in aromatic ring recorded in 3408.84 cm^{-1}. Spectra band at 1364.26 cm^{-1} support the presence of bending vibration of O-H bonds in phenol. The aromatic band for C=C stretching vibration at 1590.58 cm^{-1} is also detected. Include with stretching vibration aromatic =C-H in about 3100 cm^{-1}. All these together, resemble to the molecule of flavonoid 4',5,7-trihydroxyflavone (Figure 1). However, it requires further analysis based on spectrometry methodology to confirm the molecular structure.

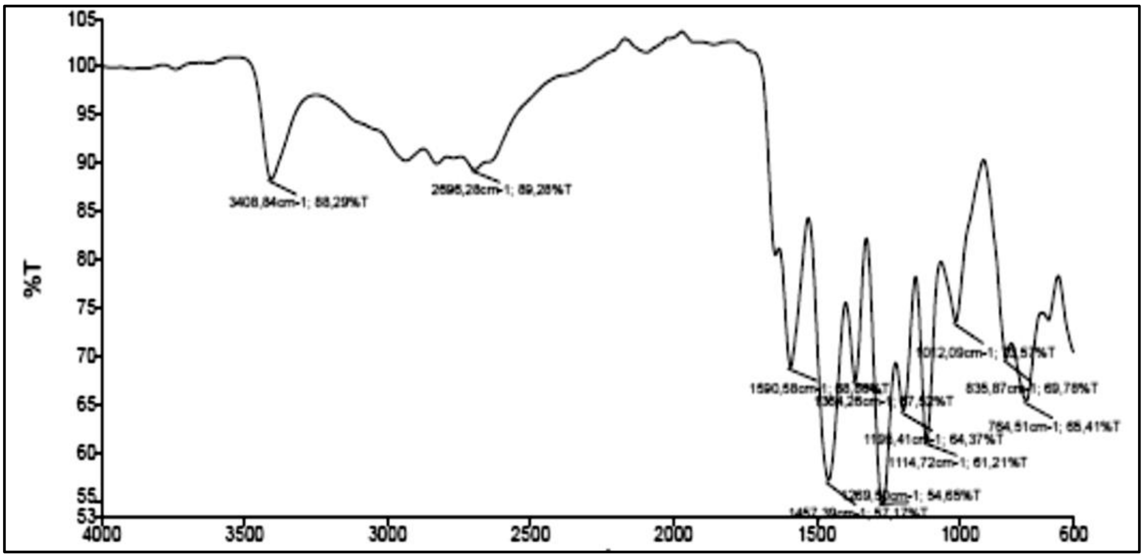

Figure 2. FTIR spectrum of fraction F2

Antidiabetic evaluation *in vivo*

Evaluation of antidiabetic in vivo was conducted using diabetic's white rat. Male mice about 20-30 months old have a similar metabolic system to humans. In addition, immune system in male mice tends not to be influenced by reproductive hormones. Thus, group of sample becomes homogeneous, easy to control, and high possibilities give more accurate data [14]. Measurement of blood sugar levels was carried out at the beginning of the treatment (H_0), after alloxan induction (H_4), and after giving the fraction (H_{14}).

Mice induced by alloxan affect metabolism of glycogen. Alloxan inhibit for insulin secretion via hampering beta cell glucose sensor of glucokinase enzyme. Thus, glucose level in blood of mice level up, and it becomes diabetes type 2 [21]. In other word, alloxan also induce the output of calcium ions from mitochondria so that it can cause the cell oxidation process to be disrupted and be the beginning of cell death so that insulin production in pancreatic cells is disrupted and can lead to diabetes mellitus [21]. As a comparison, the synthetic drug glibenclamide has been used which has been shown to reduce blood sugar levels in diabetics. This is due to the mechanism of action of glibenclamide which lowers blood glucose levels by increasing intracellular calcium in pancreatic beta cells thereby stimulating insulin production in the body. The increase in the insulin ratio causes the change in glucose into energy to increase so that it will reduce blood sugar levels [22]. While the negative control used 0.5% Na-CMC suspension which is neutral so it does not affect blood sugar levels. In the in vivo antidiabetic test, the effect of giving fractions on blood sugar levels and body weight of mice will be seen.

The evaluation result of the fraction from ethyl acetate fraction *P. cenescens* Jack injected into group of mice is summarized in Table 5 and 6. Sample F1 reduce glucose level in blood by 15.35%, F2 32.27% and F3 50.16%. Meanwhile the isolate sample is a precipitate from vial number 9 of F2 capable to reduce glucose level by 43.06%. Both F2, F3 and isolate F2 are comparable to that of glibenclamide. The data on blood sugar levels obtained were also

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statistically analyzed using the SPSS. The data groups have a significance value ($p>0.05$). Similar activity in reducing glucose level also reported previously by Tarigan et al. [23] and Syofyan et al. [24] by using methanol and ethanol extract from *P. canescens* Jack.

Table 5. Glucose level in groups of mice treated with different samples

Treatment Group	Average blood glucose levels (mg/dl) ± SD			Percentage of blood glucose levels
	H_0	H_4	H_{14}	
Control positive (K+	$110.7^{a} \pm 4.4$	$138.7^{a} \pm 5.4$	$88.7^{a} \pm 15.1$	-56.39%
Control negative (K-)	$129.0^{b} \pm 3.0$	$162.5^{b} \pm 5.5$	$170.5^{b} \pm 9.5$	+4.92%
Fraction F1	$121.7^{ab} \pm 4.3$	$160.3^{b} \pm 4.3$	$139.0^{ab} \pm 5.7$	-15.35%
Fraction F2	$113.5^{a} \pm 2.5$	$145.5^{ab} \pm 0.5$	$110.0^{a} \pm 35.0$	-32.27%
Fraction F3	$112.7^{a} \pm 2.4$	$153.7^{ab} \pm 5.5$	$102.3^{a} \pm 17.6$	-50.16%
Isolate	$128.0^{b} \pm 1.0$	$149.5^{ab} \pm 10.5$	$104.5^{a} \pm 17.5$	-43.06%

Further analysis of body weight of groups of mice is showed in Table 6. Change of body weight also give indication to reduction of body weight for diabetes mice (K-). It decreases from 31.1±0.20 mg/dL into 30.3±0.10 mg/dL. However, treatment with glibenclamide as control positive (K+) and fractions (F1, F2, F3 and isolate) indicate increment of body weight in rat model [22]. The change was also statistically evaluated One Way ANOVA test to evaluate the different change whether significance change ($p>0.05$) or not.

Table 6. Average changes in body weight of groups mice

Mice groups treated with	Average weight (mg/dL) ± SD	
	H_0	H_4
Control positive (K+	$27.7^{a} \pm 1.59$	$29.7^{a} \pm 1.82$
Control negative (K-)	$31.1^{a} \pm 0.20$	$30.3^{a} \pm 0.10$
Fraction F1	$28.6^{a} \pm 0.72$	$29.3^{a} \pm 1.28$
Fraction F2	$32.8^{a} \pm 0.35$	$32.0^{a} \pm 0.35$
Fraction F3	$30.3^{a} \pm 1.08$	$31.0^{a} \pm 0.92$
Isolate	$28.8^{a} \pm 2.00$	$30.4^{a} \pm 1.25$

The treatment group (K-, K+, F1, and isolates) was significantly different from the treatment group (F2 and F3). This is presumably due to the difference in the number of fractions given. Fraction F2 and F3 are the two groups with the highest fractions given, namely 300 mg/kgBW and 600 mg/kgBW, so that they can maintain body weight better than the treatment group (K-, K+, F1, and isolates) which had a lower dosage. The flavonoid content is predicted contribute to maintaining body weight, and in some points decrease the weight [25].

In vitro antidiabetic activity evaluation

Evaluation antidiabetic *in vitro* was carried out by measuring the capability of the sample to inhibit the performance α-glucosidase enzyme. This enzyme contributes to breaking down carbohydrate into glucose. The in vitro model test using p-nitrophenyl-α-D-glucopyranoside (PNPG) which represents carbohydrates in the body, and the result was recorded using Enzyme-Linked Immunosorbent Assay (ELISA reader) instrument. This

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substrate is hydrolyzed into glucose and p-nitrophenol. The p-nitrophenol resulted give a fading yellow color [26].

Inhibitory activity test is displayed in Table 7 and indicate the increase concentration of fraction improve capability. It was reported 0.208% inhibition at 5 ppm of sample, and it increases to 12.90% inhibition at 100 ppm of fraction. The value of IC_{50} was not determined. Comparing to the reference of acarbose [27], this result is incomparable.

Table 7. *In vitro* antidiabetic activity test

Sample	Concentration (ppm)	Absorbance	% Inhibition	IC_{50} (μG/mL)
Ethyl acetate fraction	100	1.154	-12.903	ND
	50	1.103	-8.567	
	25	1.093	-7.700	
	10	1.063	-4.821	
	5	1.016	-0.208	
Acarbose	10	0.076	95.905	0.22
	5	0.093	93.635	
	1	0.223	72.925	
	0,5	0.303	60.696	
	0,1	0.443	37.798	

CONCLUSION

In conclusion, the ethyl acetate fraction of Sungkai leaf (*P. canescens* Jack) contains flavonoid, phenolic/tannin, and steroid compounds. Indication of flavonoids composed the fraction F2 from ethyl acetate fraction. The fraction has potential to lower glucose level in diabetes model rat, and balancing capability to body weight of tested group of mice. However, in vitro evaluation using DNP substrate provide converse result. The flavonoid as is predicted composed the fraction require further investigation to confirm its molecular structure and contribution to bioactivity

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