Antidiabetic Activity of Fractions and Sub fraction of Iler [*Plectranthus scutellarioides* (L.) R. Br.] Leaves on Diabetic Mice Induced by Alloxan

Salaeh A.[1], Augusti R.S.[2], Susilawati Y.[2], Sumiwi S.A.[2] and Moektiwardojo M.[2]
1. Fatoni University, 135/8 M. 3, Khao Tum, Yarang District, Pattani, 94160, THAILAND
2. Faculty of Pharmacy Universitas Padjadjaran, Jatinangor, Sumedang, Jl. Raya Bandung-Sumedang km 21, 45363, INDONESIA

Abstract
The purpose of this research was to search bioactive compounds that have antidiabetic activity in iler leaves.The method was conducted by separation of ethanol extract by LLE, antidiabetic test of fractions, separation of most active fraction into subfractions by VLC and examine the antidiabetic activity of subfractions. The test of antidiabetic activity was used in vivo method in white male Swiss Webster mice which was induced by alloxan (i.p) and glibenclamide as positive control drug (0.7 mg/kg bw).

The results of the antidiabetic activity of the fractions at doses of 150 mg/kg bw showed that ethyl acetate (Ps-II) gave significant reduction (P<0.05) in blood glucose level (37.65%), meanwhile the n-hexane (Ps-I) and water fraction (Ps-III) did not show antidiabetic activity (P<0.05). Ethyl acetate fraction with highest antidiabetic activity was further separated by vacuum liquid chromatography column to 4 subfractions (Ps-II$_1$, Ps-II$_2$, Ps-II$_3$ and Ps-II$_4$) and showed significant antidiabetic activity (P<0.05) with percentage decrease of relative blood glucose level 44.82%, 41.67% 37.31 and 30.64% respectively. The study indicated that antidiabetic active compound of Iler leaves was present in Ps-II$_1$ and Ps-II$_2$ sub fractions, therefore it is necessary to separate the subfractions so that the structure of the active compound can be known.

Keywords: Iler, *Plectranthus scutellarioides*, Fractions, Antidiabetic, Alloxan.

Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by high glucose levels in the blood (hyperglycemia) due to defects in insulin secretion or lack of insulin sensitivity or both[1]. In general, DM is classified as type 1 DM caused by an autoimmune that causes pancreatic β-cells to be damaged so that people with lifetime insulin dependence and type 2 DM may get due to insulin resistance, lack of insulin production or both[4].Based on the International Diabetes Federation (IDF), in 2013 there are as many as 382 million people living with diabetes in the world and by 2035 that number is expected to increase to 592 million people. While in Indonesia, estimated in 2030, the prevalence of DM reached 21.3 million people[11].

Iler (*Plectranthus scutellarioides* (L.) R.Br.) is one of the medicinal plants that can be used as antidiabetic drugs. This plant can lower HbA_1C levels in diabetic rats induced by streptozotocin[5]. HbA_1C is a glycosylated hemoglobin that describes the average blood glucose level[10]. HbA_1C levels can be used to assess the quality of long-term glycemic control and assess the therapeutic efficacy, diagnosis or screening of type 2 diabetes mellitus.[7]

Iler (*P. scutellarioides*) leaves of extracts ethanol previously showed to antidiabetic activity at dose 200 mg/kg bw with percentage of relative glucose decrease of 21.52% in white rat (*Rattus novergicus*) Wistar strain induced with alloxan and glibenclamide as positive control drugs[7]. The study showed the potential of ethanol extracts of iler leaves as antidiabetic. It is necessary to know the antidiabetic active compound by separation guided by activity methods. This research will separate ethanol extracts of iler leaves into ethyl acetate fraction, *n*-hexane fraction, water fraction and will separate the most active fraction into subfractions and examine the antidiabetic activity of subfractions *in vivo*.

Material and Methods

Materials: The material used is leaves of iler (*Plectranthus scutellarioides* (L.) which has been dried obtained from Indonesian Spices and Medicinal Crops Research Institute, Bogor, West Java in October 2016.The material used was leaves of iler (*Plectranthus scutellarioides* (L.) which has been dried from Indonesian Spices and Medicinal Crops Research Institute (Balitro), Lembang, Bandung, West Java, Indonesia in October 2016. The plant was identified by biologist Mr. Joko Kusmoro (Padjadjaran University). A voucher specimen 409/HB/09/2016 has been deposited at the Herbarium of the Department of Biology, Padjadjaran University, Sumedang, Indonesia.

Animals: The experimental animals used were white male mice (*Mus musculus*) Swiss Webster strains aged 2-3 months weighing 20-30 gram.

Chemicals: The chemicals used are 70% ethanol (Bratachem), ethyl acetate (Bratachem), *n*-hexane(Bratachem), Dragendorff reagent (Sigma), Mayer reagent (Sigma), Liebermann-Burchard reagent (Sigma), H_2SO_4, Silica gel 60 (Merck, Darmstadt, Germany), Silica

gel GF_{254} (Merck, 0.25 mm), alloxan, glibenclamide, PGA 2% etc.

Apparatus: Macerators, Evaporator (Buchi), Waterbath, Separator funnel, Vacuum liquid chromatography, Glukometer (Optium Xceed), Glucose test strips (FreeStyle Optium H).

Research Method: Iler leaves were determined in *Herbarium Jatinangor*, Laboratory of Plant Taxonomy, Department of Biology,Faculty of Mathes and Science, Padjadjaran University with the document number 4393/I1.CO2.2/PL/2016. The research was agreed by the Health Research Ethics Committee of Faculty of Medicine of Padjadjaran University, with the document number 1222/UN6.C1.3.2/KEPK/PN/2017.

Extraction: Iler leaves were extracted with 70% ethanol by macerator apparatus, then kept for 3x24 hours by changing ethanol every 24 hours. Liquid extract was then separated by using flannel cloth and the filtrate was concentrated by evaporator.

Phytochemical screening of Iler: Iler extract was qualitatively tested for the presence of secondary metabolite in extract by using phytochemical screening[8].

Fractionation: Ethanol extract (30 g) was suspended in distilled water (300 mL). Then the suspension obtained was placed into a separatory funnel. First, the solution was extracted with *n*-hexane (3×300 mL). Next, the aqueous layer was extracted with ethyl acetate (3×300 mL). All of the *P. scutellarioides* fractions obtained were concentrated using the rotary evaporator. The concentrated fractions further will be referred as *P. Scutellarioides n*-hexane fraction (Ps-I),ethyl acetate fraction (Ps-II) and water fraction (Ps-III).

Subfractionation: The ethyl acetate fraction sample was performed separating the compound by Vacuum Liquid Chromatography method (VLC) using eluent *n*-hexane: ethyl acetate (10: 0, 9: 1, 8: 2, 7: 3, 6: 4, 5: 5, 4: 6, 3: 7, 2: 8, 1: 9, 0:10) and 100% ethanol and the results can be in TLC using eluent *n*-hexane: ethyl acetate (8: 2). The concentrated subfractions further will be referred as *P. scutellarioides* sub fractions ($Ps\text{-}II_1$, $Ps\text{-}II_2$, $Ps\text{-}II_3$, $Ps\text{-}II_4$ respectively).

Induction of diabetes: All Swiss Webster male white mice were acclimated for 2 weeks before the experiment. Then induce diabetic with alloxan intraperitoneally, except mice in the normal control group. After 48 hr, blood glucose was measured by glucometer.

Experimental procedure: The diabetic mice (glucose level > 140 mg/dL) were separated and divided into 6 different groups for experimental study with each group containing 4 animals. Furthermore the group of normal mice and 11 groups of diabetic mice (induction with alloxan 245 mg/kg bw, i.p) were daily administered for 7 days oral treatment.

(1) Fractions Group: 3
Group I: Normal control group, PGA 2% (not alloxan induction).
Group II: Negative control group, PGA 2%.
Group III: Positive control group, glibenclamide at dose of 0.7 mg/kg bw in PGA 2%.
Group IV: Ps-I group, Ps-I at dose of 150 mg/kg bw in PGA 2%.
Group V: Ps-II group, Ps-II at dose of 150 mg/kg bw in PGA 2%.
Group VI: Ps-III group, Ps-III at dose of 150 mg/kg bw in PGA 2%.

(2) Subfractions group:
Group I: Normal control group, PGA 2% (not alloxan induction).
Group II: Negative control group, PGA 2%.
Group III: Positive control group, glibenclamide at dose of 0.7 mg/kg bw in PGA 2%.
Group IV: $Ps\text{-}II_1$ group, $Ps\text{-}II_1$ at dose of 100 mg/kg bw in PGA 2%.
Group V: $Ps\text{-}II_2$ group, $Ps\text{-}II_2$ at dose of 100 mg/kg bw in PGA 2%.
Group VI: $Ps\text{-}II_3$ group, $Ps\text{-}II_3$ at dose of 100 mg/kg bw in PGA 2%.
Group VII: $Ps\text{-}II_4$ group, $Ps\text{-}II_4$ at dose of 100 mg/kg bw in PGA 2%.

Blood glucose measurements were performed daily from the first day of administration using an amperometric method with glucometer. From the data of blood glucose levels obtained, calculate the percentage decrease of blood glucose level relative (P) from each test group by the formula:

$$\text{Relative blood glucose (\%)} = \frac{\text{Blood glucose at t}}{\text{Blood glucose basal}} \text{ x } 100$$

$$\text{P (\%)} = \frac{\text{Relative blood glucose negative group - Relative blood glucose test group}}{\text{Relative blood glucose negative group - Relative blood normal group}} \text{ x } 100$$

Statistical analysis: The results of the study were subjected to one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. Values with $P<0.05$ were considered significant.

Results and Discussion

Dried leaves extraction (2446.38 g) resulted in 430.52 g of ethanol extracts (17.60%). Phytochemical screening of iler leaves showed the presence of alkaloids, flavonoids, saponins, polyphenols and monoterpenoids/ sesquiterpenoids (table 1). Mektiwardoyo et al[13] who reported that alkaloids, saponins, phenols, flavonoids, monoterpenoids/sesquiterpenoids and tannins were present

in the leaves of iler but in this study tannins are not detected due to the tannin content in the extract and the dried leaves. Some researchers reported that flavonoids, tannins, saponins, alkaloids and polyphenols act as bioactive antidiabetic principles. Alkaloid has α-glucosidase enzyme inhibition and decreases glucose transport through the intestinal epithelium[12,15]. Mechanism of flavonoids in lowering blood glucose levels is to reduce the absorption of glucose and increase secretion of insulin, decrease oxidative stress, inhibit the intestinal mucosa GLUT 2 and inhibit phosphodiesterase[2].

Diabetes Mellitus is a marked metabolic disease in the presence of hyperglycemia which is caused by lack of production insulin, insulin resistance, or both[3]. The induction of experimental diabetes in mice using chemicals selectively destroys pancreatic β-cells resulting in a decrease in endogenous insulin secretion and paves ways for the decreased utilization of glucose by body tissues and is simple to use as alloxan[19].

In this experiment of fractions, blood glucose levels in diabetic mices were raised nearly to 2–2.5-fold as compared to normal control group mices. The increase in glucose levels in negative control group (Diabetic control) was found to be significant (P<0.05) when compared to normal control group and basal value. Daily oral treatment with glibenclamide 0.7 mg/kg bw (Positive control) and Ps-IIat dose of 150 mg/kg bw showed significant reduction (P<0.05) in blood glucose on successive days of the experiment as compared to their basal values but Ps-I and Ps-IIIat dose of 150 mg/kg bw showed no significant reduction (P<0.05) (fig. 1).

The most active fraction for antidiabetic activity is Ps-II with the percentage decrease of blood glucose level relative (P) compared with diabetic mices showing Ps-I (9.95%), Ps-II (37.65%) and Ps-III (8.03%) at dose 150 mg/kg bw (fig 2). Results indicated that Ps-II showed the most antidiabetic activity caused by the content of flavonoid compounds suspected to provide antidiabetic activity found in the ethyl acetate fraction. Flavonoids include natural phenolic compounds that are potent antioxidants and have bioactivity capabilities as antidiabetes.

Glibenclamide orally to positive group was used to compare the antidiabetic effect. Glibenclamide is a type of medicine called sulphonylurea. It is used to help control blood sugar levels in people with type 2 diabetes. Glibenclamide works mainly by stimulating the cells in the pancreas that produces insulin, causes the beta cells to produce more insulin[18].

Most flavonoids exhibit a working mechanism as antidiabetic by the pancreas[9]. In addition to flavonoids, saponins are also thought to reduce blood glucose levels by working like insulin that can stimulate glucose uptake by muscle cells. The mechanism of action of saponins is the same as the class of antidiabetes sulfonylurea that is by inhibiting the K-ATP channel so that the potassium flow out of the cell is disrupted. The result is depolarization of pancreatic β-cell membranes so that the Ca^{2+}-ATPase channel opens and the calcium ions flow into the cytoplasm. The presence of these calcium ions activates the calmodulin enzyme in the cell resulting in the excitosis of insulin from the vesicles to be excreted out of the cell.[16]

Subfractionation of the ethyl acetate fraction (The most active fraction) by VLC method gave 12 Ps-II sub fractions. Sub fractions with similar TLC profile then combined and gave 4 Ps-II sub fraction ($Ps\text{-}II_1$, $Ps\text{-}II_2$, $Ps\text{-}II_3$ and $Ps\text{-}II_4$). Antidiabetic activity was further tested.

In the sub fractions, blood glucose level was measured in all groups showing that there was a treatment effect in each group. Data showed that negative control mice gave a significant elevation (P<0.05) in blood glucose of the experiment as compared to their basal values maintained over a period of 7 days although it decreased but still in diabetic condition. Daily oral treatment with glibenclamide 0.7 mg/kg bw and subfraction at dose of 100 mg/kg bw showed significant reduction (P<0.05) in blood glucose on successive days of the experiment as compared to their basal values (fig. 3).

Table 1
Phytochemical screening of iler

Secondary metabolite	Dried leaves	Concetrated Extract
Alkaloids	+	+
Polyphenols	+	+
Tannins	-	-
Flavonoids	+	+
Quinons	-	-
Saponins	+	+
Monoterpenoids/Sesquiterpenoids	+	+
Steroids/Triterpenoids	-	-

Note: + : Detected - : Undetected

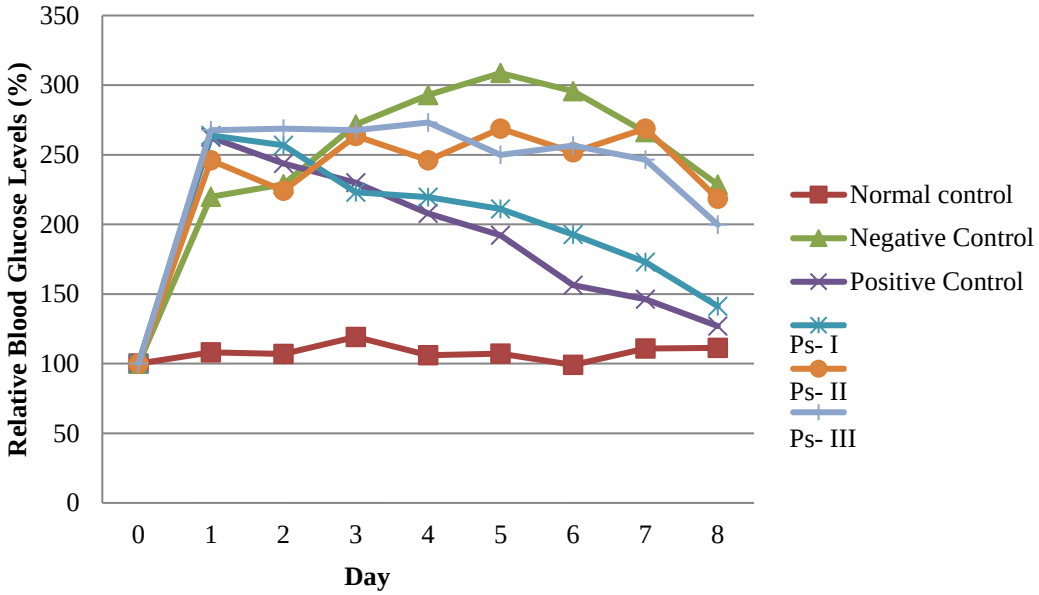

Fig. 1: Relative blood glucose levels (%) of iler fraction on mice during antidiabetic activity test
Fractions was administered at dose of 150 mg/kg bw.
Blood glucose value is mean±SEM of 4 observation,* p<0.05 compared with their basal values of respective group

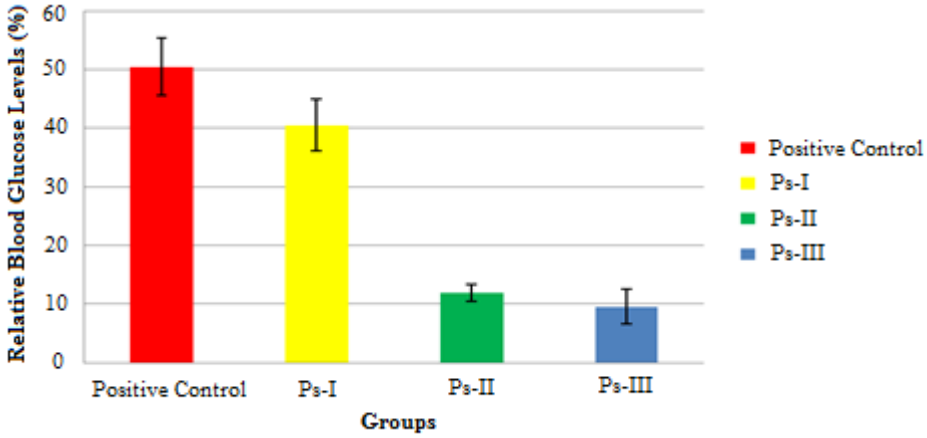

Fig. 2: Average decrease of relative blood glucose levels (%) of Iler fraction on mice against negative control
Iler subfractions was administered at dose of 100 mg/kg bw.
Blood glucose value is mean±SEM of 4 observation,* p<0.05 compared with their basal values of respective group.

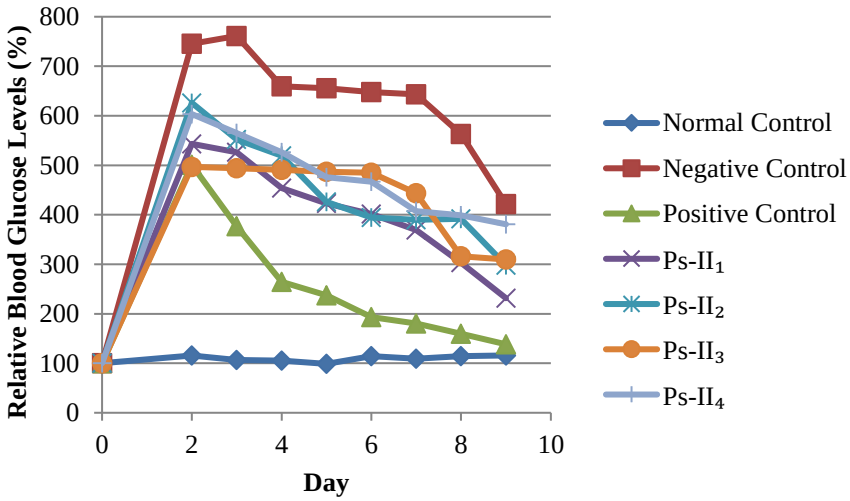

Fig. 3: Relative blood glucose levels (%) of iler subfraction on mice during antidiabetic activity test

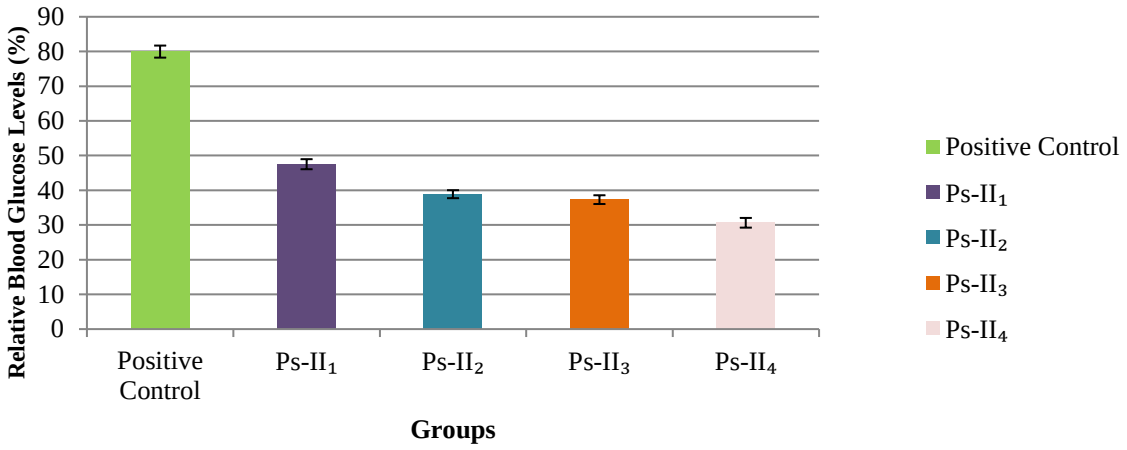

Fig. 4: Average decrease of relative blood glucose levels (%) of Iler subfraction on mice against negative control

The most pronounced antidiabetes activity was obtained by Ps-II_1 with dose of 100 mg/kg bw with the percentage decrease of blood glucose level relative (P) compared with diabetic mices showing Ps-II_1 (44.82 %), Ps-II_2 (41.67%), Ps-II_3 (37.31%) and Ps-II_4 (30.64%). Results indicated that Ps-II_1 showed the most antidiabetic activity, but not significant with Ps-II_2 (fig. 4). Subfraction probably contained several secondary metabolites as flavonoids, saponins, alkaloids and polyphenols as bioactive antidiabetic principles[14]. Flavonoids have been reported to suppress glucose level significantly and the typical flavonoid has been found to be a strong inhibitor of α-glucosidase.[8]

Conclusion

In conclusion, the present data suggest that the ethyl acetate fractions and subfraction of ethyl acetate fraction iler (*Plectranthus scutellarioides* (L.) R.Br.) leaves has good antidiabetic activity. Ethyl acetate fraction (Ps-II) is the most active fraction for antidiabetes with the percentage decrease in blood glucose level relative of 37.65% at dose of 150/kg bw. Subfraction 1 of the ethyl acetate fraction (Ps-II_1) had the most active antidiabetic activity at dose of 100 mg/kg bw with a percentage decrease of blood glucose level relative 44.82% in white male mice (*Mus musculus*).

Swiss Webster strains induced by alloxan and glibenclamide drug served as a positive control. The research indicated that antidiabetic active compounds of Iler leaves contained in Ps-II_1 and Ps-II_2 sub fractions are semipolar compound. It is necessary to separate the subfractions so that the structure of the active compound can be known.

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