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Antidiabetic Activity of Active Fractions of Leucaena Leucocephala (lmk) Dewit Seeds in Experiment Model

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Abstract

A study was conducted on antidiabetic test of active fractions of methanol extract from *Leucaena leucocephala* (lmk)DeWit seeds using alloxan-induced rats. Fractionation was conducted on active fractions using column chromatograph. The most active fractions in the previous study were analyzed with thin layer chromatography (TLC) using mobile phase of chloroform-methanol (5:1), chloroform–methanol (2: 1), chloroform-methanol-water (5: 5: 1) on the isolates yielded with oral glucose tolerance test and identification. The result findings show that methanol extracts have a greater antidiabetic activity; and 5 isolates resulted from the isolation of methanol extracts. The result of bioactive compound identification was glycoside compounds with monosaccharide galactose clusters and many other saccharides. It was concluded that active fractions of *Leucaena leucocephala* (lmk)DeWit seeds had antidiabetic activities and their bioactive compounds constitute glycoside compounds with monosaccharide galactose clusters and many other saccharides.

Keywords: *L. leucocephala* (lmk)DeWit seeds, antidiabetes, alloxan, oral glucose tolerance

1. Introduction

Diabetes mellitus is a common degenerative disease among the communities with different age groups and different socioeconomic levels. Syndromes of diabetes mellitus include chronic hyperglycemia that is a manifestation of abnormalities in carbohydrate, protein, and fat metabolisms or is correlated with the deficiency of insulin secretion within the target cell membranes (Alberti and Zimmeti, 1998).

The incidence of diabetes keeps increasing annually. In 2000, the number of diabetic patients in Indonesia was up to 8.4 millions patients and ranked the fourth worldwide after India, China, and the United States of America (Wild *et al*., 2004) Similarly, data obtained from 7 hospitals in Jakarta

(RSCM, Fatmawati, Cikini, Pelni, Persahabatan, Husada and St. Carolus) showed that the number of visits at outpatient services was the greatest for diabetes (Anonym, 2000)

Recently, more than 400 types of plants have been reported to be useful as alternative and complementary treatments for diabetes; however, relatively few had been studied in terms of their natural efficacy (Lee *et al*., 2006). One of the plants recently used as an alternative and complementary treatment for diabetes is *L. leucocephala* (lmk)DeWit. The plant has been used by human beings since many centuries ago for herbal medicine. Indonesia is one of the tropical countries, which are rich of natural resources. Forest biodiversity as a natural resources remains in need of more exploration to recognize its potentials as a source of herbal medicine, including for antidiabetes (Syamsudin *et al*., 2006). According to the priority of the study, development and implementation of health science and technology for medicine and health devices for the period of 2005-2025 have included standardized herbal products and phytopharmaca. The recent study aims at isolating bioactive compounds from *L. leucocephala* (lmk)DeWit seeds as an anti-diabetes.

2. Materials and Methods

2.1. Plant Material

L. leucocephala (lmk)DeWit seeds were obtained from BALITRO Bogor and then determined in Herbarium Bogoriense, Bogor. The experimental models used in the study were rats (*Mus musculus*) aged 3-4 months.

2.2. Extraction and Fractionation

L. leucocephala (lmk)DeWit seeds were grounded to powder; then refluxed three times in a gradient way using solvents like *n*-hexane, ethylacetate, methanol, and water as well as direct extraction with methanol. Each extract is let to evaporate and held until fractions are yielded.

2.3. Hypoglycemic Activity Tests

Before treated, the rats were injected with alloxan with a dosage of 70 mg/kg body weight *in vitro*. Subsequently, blood samples were taken to figure out the hyperglycemic levels of the experimental models. Once the rats were hyperglycemic, they were divided into 6 groups of hyperglycemic rats and 1 group of normal rats as a control group; each group contained 6 rats.

(1) Normal control group: a group of rats which were not treated and were normally fed;
(2) Negative control group: A group of hyperglycemic rats, which were treated with distilled water of 2 mL orally for 14 days;
(3) Group A: A group of hyperglycemic rats which were treated with *n*-hexane extract with an oral dosage of 0.5 g/kg body weight on daily basis for 14 days.
(4) Group B: A group of hyperglycemic rats which were treated with ethylacetate extract with an oral dosage of 0.5 g/kg body weight on daily basis for 14 days.
(5) Group C: A group of hyperglycemic rats which were treated with indirect methanol extract with an oral dosage of 0.5 g/kg body weight on daily basis for 14 days.
(6) Group D: A group of hyperglycemic rats which were treated with water extract with an oral dosage of 0.5 g/kg body weight on daily basis for 14 days.
(7) Group E: A group of hyperglycemic rats which were treated with direct methanol extract with an oral dosage of 0.5 g/kg body weight on daily basis for 14 days.
(8) On Day 0, 3, 7, and Day 14 blood samples were taken through tail vein; the level of blood glucose was then measured using a glucometer.

2.4. Purification with Column Chromatography

Methanol extracts were fractionated with column chromatography using chloroform-methanol eluents in a gradient way, ranging from (5:1), (4:1), (3:1), (2:1), and (1:1) to categorize the compounds contained in the methanol extracts based on their polarity.

2.5. Oral Glucose Tolerance (OGT) Method

With this method, 35 rats were initially acclimatized before the experimental phase. The rats were grouped into seven, each with 5 rats.

(1) Negative control group: a group that was only treated with oral suspension of CMCNa 0,1% with a volume of 0.1 mL.
(2) Positive control group: a group that was only treated with oral quercetin with a dosage 10 mg/kg body weight. An hour after the treatment, the rats were given glucose solution with a dosage of 1,5 mg/kg body weight.
(3) Isolate Group A-1: a group which was treated with oral A-1 isolation solution with a dosage of 10 mg/kg body weight. An hour after the treatment, the rats were given glucose solution with a dosage of 1,5 mg/kg body weight.
(4) Isolate Group A-2: a group which was treated with oral A-2 isolation solution with a dosage of 10 mg/kg body weight. An hour after the treatment, the rats were given glucose solution with a dosage of 1,5 mg/kg body weight.
(5) Isolate Group A-3: a group which was treated with oral A-3 isolation solution with a dosage of 10 mg/kg body weight. An hour after the treatment, the rats were given glucose solution with a dosage of 1,5 mg/kg body weight.
(6) Isolate Group A-4: a group which was treated with oral A-4 isolation solution with a dosage of 10 mg/kg body weight. An hour after the treatment, the rats were given glucose solution with a dosage of 1,5 mg/kg body weight.
(7) Isolate Group A-5: a group which was treated with oral A-5 isolation solution with a dosage of 10 mg/kg body weight. An hour after the treatment, the rats were given glucose solution with a dosage of 1,5 mg/kg body weight.

An hour following the administration of preparations (hour 0), blood samples of the rats were immediately taken through their tail vein. Blood taking were repeated with an interval of 0.5 hour starting from hour 0 through hour 2,5 using a glucometer.

2.6. Identification of Active Compounds

Purified isolates were identified using a spectrophotometer UV-Vis, IR and RMI.

2.7. Data Analysis

The data were initially tested for their normality and homogeneity. When the data were normally distributed and homogenous, they were further analyzed with one-way ANNOVA using a level of significance $p = 0.05$ and 0.01. When a significant difference was noted, the further step was Turkey test to find out the existence of real difference among the treatment groups.

3. Results and Discussion

Obtaining animal models with diabetes mellitus could be done with pancreatomy and administration of certain chemical substances. The chemical substances used were allocsan due to its greater selectivity on rats compared to mice within the merusal of pancreatic cell β. In addition, allocsan increases the hyperglycemic effect within 2 to 3 days. Before used for experimental purposes, all rats from each

group were measured for their body weight and subsequently taken care for a week. The result of body weight measurement is presented in the following Table 1.

Table 1: Mean body weights of rats after the treatment with test preparations

	normal	**diabetes**	***n*-hexane**	**ethylacetae**	**methanol A**	**water**	**methanol B**
Baseline	31.7 ± 2.4	32.4 ± 2.4	34.1 ± 3.4	30.4 ± 4.5	36.8 ± 3.2	37.6 ± 3.9	35.0 ± 6.4
Day-0	32.8 ± 4.3	24.8 ± 2.8	28.3 ± 4.2	24.7 ± 6.4	32.2 ± 6.3	33.2 ± 8.3	29.9 ± 4.2
Day -3	33.9 ± 5.4	26.2 ± 4.3	29.2 ± 4.3	25.1 ± 3.4	34.2 ± 4.2	34.9 ± 3.8	31.1 ± 4.5
Day-7	34.7 ± 3.2	26.9 ± 4.3	30.1 ± 5.7	27.6 ± 7.3	34.1 ± 5.7	35.7 ± 5.4	32.5 ± 6.9
Day-14	35.8 ± 4.3	28.4 ± 2.5	30.2 ± 6.4	28.1 ± 4.8	35.2 ± 8.6	36.5 ± 7.8	33.3 ± 5.4

Table 1 shows that on Day 0, body weights of hyperglycemic states of rats in control group, treatment groups *n*-hexane and ethylacete decreased. It may be due to abnormalities in glucose metabolism in which energy supply were not sufficient, causing depletion of fatty cells and protein in order to meet energy requirements that could be sufficiently supplied from glucose metabolism. On Day 14, body weights of all rats in the treatment groups were restored even though they had not gained their baseline body weight. This was perhaps since energy had been sufficiently supplied and glucose metabolism had been adequate. Results of observations on amounts of feed, drinking water volume, and urine volume are presented in Table 2.

Table 2: Mean drinking water volume, urine volume, and amounts of feed

Groups	**Drinking Water Volume (mL)**	**Urine Volume (mL)**	**Amount of feed (g)**
Normal	7.60 ± 1.23	1.10 ± 0.92	3.67 ± 1.92
Diabetes	13.98 ± 3.41	3.46 ± 1.42	11.37 ± 1.32
n-hexane	7.80 ± 1.42	2.40 ± 1.31	5.37 ± 2.41
ethylacetate	7.51 ± 1.34	2.44 ± 0.98	5.24 ± 1.52
methanol A	6.14 ± 2.41	2.14 ± 1.34	4.73 ± 1.31
water	5.92 ± 2.45	2.06 ± 1.45	4.37 ± 2.34
methanol B	5.98 ± 0.89	1.89 ± 1.03	4.95 ± 7.59

Table 2 shows that in diabetic groups, drinking water volume and urine volume were greater than those in normal and treatment group. It was probably attributable glucosaria among the diuretic osmotic hyperglycemic groups; hence resulting in dyuresis followed by a depletion of electrolyte since the body tried to overcome the dyuresis by drinking more (polydipsia). Amount of feedings among diabetic groups increased more greatly (polyphagia) than those among normal and treatment groups. This is perhaps resulted from stimulation in the center of appetite within the hypothalamus because of insufficiency in the utilization of glucose within the cells due to hyperglycemia. Syndromes like polyuria, polydipsia, and polyphagia were commonly found among diabetic. After the extract administration to all treatment group A-E, an increase in the level of blood glucose was observed both on Day 3, Day 7, and Day 14.

Figure 2: Potential decrease in the level of blood glucose among all extract groups

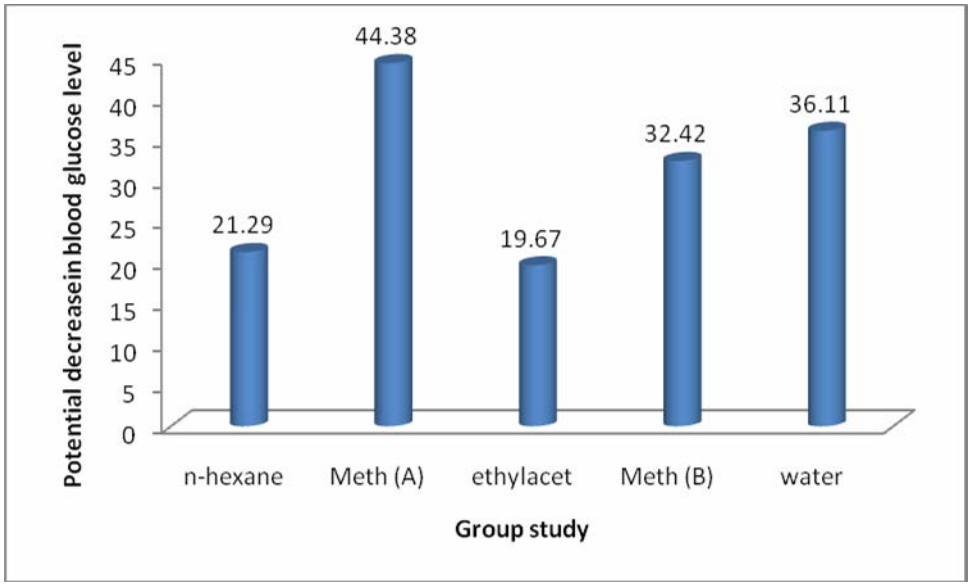


Figure 2 shows that group direct methanol extract (meth B) had greater decrease in the level of blood glucose than any other groups, i.e. a decrease of 44.38%, *n*-hexane of 21.29%, ethylacetate of 19.67%, indirect methanol extract of (meth A) 32.42%, and water extract of 36.11%. Group meth B and water had the greatest capability in decreasing the level of blood glucose compared to any other groups. This was perhaps because group meth B and water constituted polar solvent and contained compound groups that are found in indirect methanol extracts (meth A) and water extract. It was estimated that groups with the greatest capability of reducing the level of blood glucose were groups with polar solvent. Table 3 presents rendement or yields of each extract.

Table 3: Rendements of each extract

Extract	Rendement (%)
n-hexane	2.19%
ethylacetate	1.14%
methanol	5.42%
water	5.67%
direct methanol	6.32 %

Table 3 shows that rendement extract with polar solvent like methanol, water, and direct methanol were more considerable than non-polar solvents (n-hexane) and semi-polar solvents (ethylacetate). Graded extractions were conducted by using solvents with different polarity. The objective was for initial pre-fractionation since it could isolate chemicals properties contained in the *L. leuco cephala* (lmk)DeWit seeds based on their polarity.

Fractionation with column chromatograms aimed at isolating compounds within the methanol fractions with the expectation that a pure compound could be obtained. Methanol extract at preliminary test was subsequently fractionated with column chromatography by using appropriate eluents. Fractionation was done with chloroform-methanol eluents in a gradient way with respective solvent proportions of 5:1, 4:1, 3:1, 2:1, 1:1. Eluate from each extract was retained well. Every fraction was also treated with TLC. Fractions with similar pattern of isolation with chromatograms were combined; yielding simpler fractions. The results of combining TLC examination could be summerized as shown in the following Table 4.

Table 4: The Result of combining TLC examination of all Fractions

Sub-fraction	Combination	spot color	Number of spots	Rf
A-1	1-10	brown	1	0.88
A-2	11-17	tosca green	1	0.48
A-3	13-27	brown tosca green brown	3	0.29 0.19 0.097
A-4	37-60	bright green dark green brown	3	0.25 0.19 0.097
A-5	61-90	dark green	1	0.19
A-6	91-137	dark green	1	0.16

In relation to 6 sub-fractions that had been obtained, oral glucose tolerance test was conducted to rats treated with glucose in an oral dosage of 1.5 g/kg body weight; then, the level of blood glucose was measured through tail vein on minutes 0, 30, 60, 90, 120, and 180 using glucometer. To find out their potentials in reducing the level of blood glucose, the size of area under the curve was measure to be subsequently used to figure out its ability to reduce the level of blood glucose in experimental rats following the administration of test preparation. The results are presented in Figure 3.

Figure 3: Diagram of the potential decrease in the level of blood glucose from test preparations

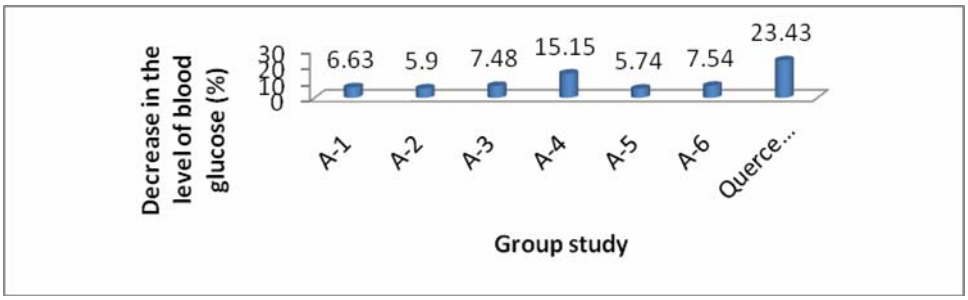
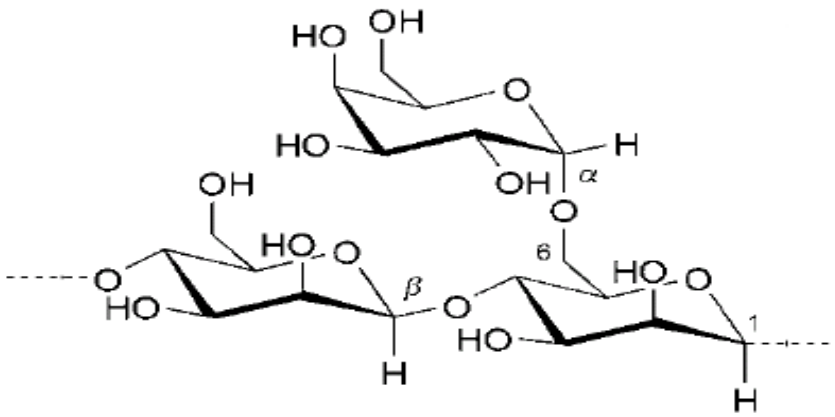


Figure 3 shows that the potential decrease in the level of blood glucose of isolate A-4 is higher (15.15%) than isolate A-1 (6.63%), A-2 (5,90%), A-3 (7.48%), A-5 (5,74%) and A-6 (7.54%). Statistical analysis shows no significant difference between quercetin (positive control) and isolate A-4 in reducing the level of blood glucose ($p>0.05$). Isolate A-4 or active isolates were purified with High-Performance Liquid Chromatography (HPLC) using the column of reversed phase C_{18}, with mobile phase of methanol-water (5:1); hence pure isolate compound could be obtained. In the chromatogram of HPLC, in the isolate A-4 were observed two main peaks; and the sharpest one is peak 2 which in on 16.876 minutes. Then, isolates within the minute were retained based on the chromatogram detected by HPLC. Purification was done with repeated injection. Pure isolate was subsequently identified. Investigation on the spectrum of isolate compounds A-4 with proton RMI showed that chemical shift between 0,88 bpj ~ 1,27 was estimated to contain single bonded CH_3- ; -CH_2- dan -CH-clusters. An anomeric proton of a glycoside was found at δH 3, 20 ~ 4,47 bpj and 5,37 bpj. Investigation on the spectrum of isolate compounds A-4 on carbon RMI indicated that the signals of isolate compounds A-4 had 40 carbon atoms. Chemical shift at δC 83,84 bpj ~ 105,37 bpj indicated the existence of glycoside; meanwhile, chemical shift at δC 54,79 ~ 79,67 bpj indicated the existence of oxygenated carbons. Therefore, based on the data isolate A-4 could only be predicted as a glycoside compound with galactose monosaccharide clusters and other saccharides.

Figure 4: The structure of galactomannan



In another study on *L. leucocephala* (lmk)DeWit seeds, it was indicated that *L. leucocephala* (lmk)DeWit seeds contain galactomannan and lectin galactomannan that constitutes a glycoside (Lesniak and Liu, 1981). A study conducted by Ali et al. (1995) on antidiabetic test on active fractions of *Trigonela foenum graecum* seeds of Leguminosae family showed that the plants might reduce the level of blood glucose in streptozotozin-fed rats. The results of identification on molecular structures assumed to be responsible for the antidiabetic effects were galactomannan. Galactomannan were mostly dispersed within the plants of Leguminosae family. Based on the similarity among molecular formulas, it could be temporarily assumed that the molecular structures of isolate A-4 and galactomannan resulted from the isolation of *Trigonela foenum graecum* seeds, it is possible that on isolate A-4 the one responsible for the antidiabetic activity of *Leucaena leucocephala* (lmk)DeWit seeds was galactomanna. It is highly possible due to a similarity in the chemical structures of isolate A-4 and galactomannan.

4. Conclusion

Based on the findings of the study, it can be concluded that *Leucaena leucocephala* (lmk)DeWit seeds have an effect for reducing the level of blood glucose. The results of identification on the bioactive compounds indicate that glycoside compounds have galactose monosaccharide clusters and other saccharides.

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