The effect of n-butanol fraction of gaharu (*Aquilaria microcarpa* Baill.) leaves on blood glucose and liver glycogen levels in alloxan-induced male rats

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Abstract. Gaharu leaves (*Aquilaria microcarpa* Baill.) empirically used to lower blood glucose levels by people of Tamiang Layang Central Kalimantan. This study measured the activity of *A. microcarpa* leaves to blood glucose and glycogen liver levels. The study used 24 male white rats. Animal test were divided into 6 groups (n=4) receiving different treatments: normal control, negative control (vehicle), positive control (glibenclamide 0.45 mg/kg BW), n-butanol fraction (12.5 mg/kg BW; 25 mg/kg BW; and 50 mg/kg BW). All groups, excluding the normal group, were induced by alloxan monohydrate (150 mg/kg BW, i.p.). After 72 hours, the levels of glucose blood were measured and the treatment was administered. Pre-prandial and postprandial measurement of blood glucose levels were conducted on days 0, 7, and 14 after treatment. On day 15 the liver was extracted out and analyzed by UV-Vis spectrophotometry to determine the glycogen levels. The results of this study showed that blood glucose levels reduction and liver glycogen levels enhancement of n-butanol fraction of *A. microcarpa* leaves at the doses of 12.5; 25 and 50 mg/kg BW significantly different with the negative control (p < 0.05). This result suggests n-butanol fraction of *A. microcarpa* leaves has potential as an antidiabetic agent.

Keywords: *Aquilaria macrocarpa* Baill., blood glucose levels, glycogen, alloxan, rat.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by increased blood glucose levels exceeding normal levels, which is caused due to lack of insulin. Clinical symptoms that commonly occur in diabetics are high frequency of eating, drinking, and urinating as well as weight loss [1]. The International Diabetes Federation (IDF) states that the prevalence of DM in the world is 1.9% and has made DM the 7th leading cause of death in the world, while in 2012 the incidence of DM in the world was 371 million people [2]. In 2002, the number of people with DM in Indonesia ranked 4th [3].

DM treatment can be done using pharmacological therapy such as insulin and or oral hypoglycemic agents [4]. In addition to pharmacological treatment, DM patients often use herbal or plant-derived treatments. One of the plants that is efficacious as a DM treatment is the gaharu plant (*Aquilaria microcarpa* Baill.). People of Tamiang Layang Central Kalimantan empirically use the leaves of *A. microcarpa* as antidiabetic by brewing the leaves with hot water and drinking it. *Aquilaria microcarpa* leaves are known to contain several secondary metabolites such as flavonoid compounds, tannins, and phenols [5,6].

Some plant metabolites such as flavonoids, tannins, and phenols have a role in lowering blood glucose levels. Flavonoid compounds are known to play a role in reducing blood glucose levels, in addition, tannin and phenol compounds also have the potential to lower blood glucose levels [7]. Tannins
are able to increase glucose transport by activating insulin and lowering blood glucose levels [8]. Phenol has antioxidant activity that can inhibit pancreatic cell damage [9].

The activity of reducing blood glucose levels of *A. microcarpa* leaves was still limited to the test of the extract. The ethanol extract of *A. microcarpa* leaves has been shown to have efficacy as a lowering of blood glucose levels in alloxan-induced rats [5,6]. Tests on the fraction of the leaf extract of *A. microcarpa* need to be done. The fractionation process is expected to be able to separate the active compounds into more specific groups, especially flavonoids, tannins, and phenols. Fractionation is carried out using *n*-butanol as solvent, because *n*-butanol solvent is polar which can attract polar groups of flavonoids, tannins, and phenols. Flavonoids are polar compounds because they have a number of hydroxyl groups, as well as tannins and phenolic compounds that are polar so they are easily soluble in *n*-butanol solvent [10,11].

Testing the activity of lowering blood glucose levels can be observed with several parameters including blood glucose levels and liver glycogen. The normal mechanism is that liver glycogen is able to maintain glucose levels, an increase in blood glucose levels is followed by a decrease in glucagon, then glucose will be stored as glycogen [12]. Glycogen production will reduce blood glucose levels [13]. This study aimed to evaluate the activity of reducing pre-prandial and postprandial blood glucose levels and increasing liver glycogen levels of the *n*-butanol fraction of *A. microcarpa* leaves against alloxan-induced male rats.

2. Materials and Methods
2.1. Sample collection
Leaf samples were taken from *A. microcarpa* cultivation in Tamiang Layang, Central Kalimantan. Wet sortation and washing were carried out on the collected *A. microcarpa* leaves to separate impurities and other plant parts that were not needed. Drying is done by win dry and protected from direct sunlight [14]. Dry sortation is carried out on the sample to separate foreign organic matter due to the drying process. The sample was mashed using a blender to form a powder and sieved with a sieve number 25 [15].

2.2. Preparation of *n*-butanol fraction
A total of 100 grams of *A. microcarpa* leaves dry powder were extracted by continuous percolation method using 70% ethanol solvent in a ratio of 1:15 [16]. The solvent was evaporated using a vacuum rotary evaporator at a temperature of 60°C, then concentrated using a water bath at a temperature of 70°C to form a thick extract [17]. Fractionation was carried out using *n*-hexane, ethyl acetate and *n*-butanol as solvents. The thick extract was suspended using distilled water in a ratio of 1:2, then put into a separating funnel. 50 mL of *n*-hexane solvent was added and allowed to stand until two layers were formed 3 times. The water layer was again fractionated with 50 mL of ethyl acetate as solvent. The insoluble ethyl acetate layer was fractionated with 50 mL of *n*-butanol. The top layer formed, namely the *n*-butanol layer, was then separated, collected, evaporated and dried in a water bath at 70°C until the constant weight.

2.3. Experimental design
A total of 24 rats were divided into 6 groups, each group consisting of 4 rats [18]. Test animals before being given treatment were adapted for one week. All groups were fasted for 12 hours and then determined fasting blood glucose levels. All groups (except the normal group) were induced with alloxan intraperitoneally at a dose of 150 mg/kg BW. Measurement of fasting blood glucose levels was carried out on the 3rd day after alloxan induction. Test animals with blood glucose levels above 200 mg/dL were used for further research [19]. The test group was given treatment for 14 days as shown in Table 1.
Table 1. Animal test grouping.

| Group           | Treatment                                      |
|-----------------|------------------------------------------------|
| Normal group    | Na CMC 0.5% (not induced with alloxan)         |
| Positive control| Glibenclamide 0.45 mg/ kg BW                   |
| Negative control| Na CMC 0.5%                                    |
| Dose I          | n-butanol fraction of *A. microcarpa* 12.5 mg/kg BW |
| Dose II         | n-butanol fraction of *A. microcarpa* 25 mg/kg BW |
| Dose III        | n-butanol fraction of *A. microcarpa* 50 mg/kg BW |

2.4. Blood glucose levels measurement
Measurement of blood glucose levels was carried out on days 0, 7, and 14 after treatment using glucometer stick (GlucoDr®). Blood sugar levels were measured after the test animals were fasted for 12 hours (pre-prandial blood glucose levels). After that, the test animals in each group were given glucose as much as 1.75 g/kg BW orally. Measurement of blood glucose levels was carried out again after 2 hours later (postprandial blood glucose levels).

2.5. Measurement of liver glycogen levels
Bovine liver glycogen solution was prepared with a series of concentrations of 10, 20, 30, 40, 50, and 60 ppm for the standard curves. As much as 0.3 mL of each concentration series was added with 0.6 mL of 5% phenol reagent, and 3 mL of concentrated H2SO4. The mixture was incubated for 14 minutes. The absorbance was measured at a wavelength of 492 nm using a UV-Vis spectrophotometer [20].

A total of 25 mg of dry rat liver powder was extracted with 1 mL of 30% KOH solution and incubated in a boiling water bath for 20 minutes. After cooling, 1.5 mL ethanol 95% was added and allowed to stand for 30 minutes. The glycogen precipitate in the sample was separated by centrifugation at 2500 rpm for 20 minutes [21]. Sample was taken of 0.3 mL, reacted with 0.6 mL of 5% phenol reagent and 3 mL of H2SO4, then the absorbance was measured using UV-Vis spectrophotometry at a wavelength of 492 nm [22].

2.6. Data analysis
Data on pre-prandial and postprandial blood glucose levels were analyzed using ANOVA test with 95% confidence level followed by Post-Hoc LSD test. Liver glycogen level data were analyzed by Kruskal-Wallis test and Mann-Whitney test with 95% confidence level.

3. Results and Discussion
3.1. Alloxan induction
The rats were adapted for 1 week so that the rats could interact with the surrounding environment and not cause stress to the rats before being given treatment. Rats were used as test animals because rats were easier to take blood samples to measure blood glucose levels. Alloxan induction was carried out to produce diabetic conditions in rats, before induction the rats were fasted for 16 hours so that blood glucose was stable and there was no change in blood glucose levels due to food intake [19]. Fasted animals are also more sensitive to alloxan administration [23].

Alloxan is a diabetogenic compound that is cytotoxic in the body which can produce free radicals that increase oxidative stress and damage pancreatic cells so that insulin production can be reduced and cause hyperglycemia conditions [24]. Alloxan will be reduced in the body to dialuric acid which forms superoxide radical compounds. Superoxide radical compounds will cause a large increase in calcium concentration resulting in disruption of homeostasis, which causes damage to pancreatic cells [23]. The mice were then induced with alloxan intraperitoneally at a dose of 150 mg/kg BW [19]. The intraperitoneal route was chosen so that alloxan can be absorbed more quickly and reach the pancreas.
immediately, besides that this intraperitoneal route is easier to do than other routes [23]. Blood glucose levels of rats after alloxan induction are shown in Figure 1.

![Figure 1. Blood glucose levels of rats after alloxan induction.](image)

The positive control in this study was glibenclamide. The mechanism of action of glibenclamide stimulates insulin release by pancreatic cells and increases insulin binding to target tissues [25]. Glibenclamide blocks K⁺ channels in pancreatic cells, resulting in depolarization which causes Ca²⁺ channels to open, more Ca²⁺ entry into cells will stimulate insulin release [4].

### 3.2. Blood glucose levels measurement
Measurement of blood glucose levels was carried out on days 0, 7, and 14 using a glucometer [26]. Measurement of blood glucose levels is done by measuring pre-prandial and postprandial blood glucose levels. Measurement of pre-prandial glucose levels was carried out after the animals were fasted for 12 hours. The results of measuring blood glucose levels obtained in this study was varied. Factors that affect variations in blood glucose levels obtained include internal factors from test animals such as insulin receptor conditions, hormonal conditions, the condition of the pancreas of test animals during treatment [27]. The results of measuring blood glucose levels are used to determine the condition of decreasing blood glucose levels after treatment.

| Group           | Reduction rate (%) (Mean±SEM) | Day 0-7 | P value | Day 0-14 | P value |
|-----------------|-------------------------------|---------|---------|----------|---------|
| Normal group    | 0.99±3.09                     | 0.771   |         | 8.81±7.15| 0.524   |
| Positive control| 3.01±1.80                     | -       |         | 4.59±2.09| -       |
| Negative control| 50.95±2.75*                   | 0.000   |         | 60.36±3.06*| 0.000  |
| Dose I          | 43.64±2.38*                   | 0.000   |         | 59.78±4.33*| 0.000  |
| Dose II         | 47.89±10.92*                  | 0.000   |         | 62.28±2.13*| 0.000  |
| Dose III        | 51.28±2.65*                   | 0.000   |         | 64.54±3.75*| 0.000  |

Description: Pre-prandial day 0-7* p < 0.05; significantly different with negative control
Pre-prandial day 0-14* p < 0.05; significantly different with negative control
The decrease percentage of glucose levels in the negative control was not significantly different with normal group (Table 2). This is because the negative control was only given Na-CMC which had no effect as an antidiabetic. This is much different from the group with a dose of 12.5 mg/kg BW and a dose of 25 mg/kg BW on day 14 experienced a decrease in blood glucose levels as well as the positive control. The results of the percentage reduction in the dose of 50 mg/kg BW had the highest percentage decrease, so it can be concluded that the higher the dose given, the higher the decrease in blood glucose levels.

Measurement of postprandial blood glucose levels was carried out 2 hours after being given glucose intake. Under normal conditions, blood glucose levels 2 hours after eating are higher than fasting glucose levels, postprandial hyperglycaemic states occur due to imbalanced pancreatic function disorders [28]. The purpose of measuring postprandial blood glucose levels is to determine instantaneous glucose homeostasis [27]. Measurement of blood glucose levels was carried out 2 hours after eating because blood glucose levels would increase after being given glucose intake, this increases stimulated pancreatic cells to secrete insulin. Insulin secretion occurs in two stages, in the first stage insulin increases one hour after glucose administration and again decreases within 2 hours of glucose administration [29].

**Table 3. Reduction rate of post-prandial blood glucose levels.**

| Group            | Reduction rate (%) (Mean±SEM) |
|------------------|-------------------------------|
|                  | Day 0-7 | P value | Day 0-14 | P value |
| Normal group     | -3.72 ± 9.23 | 0.611 | 1.58 ± 6.72 | 0.721 |
| Positive control | 1.17 ± 0.64 | - | 4.05 ± 1.99 | - |
| Negative control | 42.22 ± 8.76* | 0.000 | 52.69 ± 5.39* | 0.000 |
| Dose I           | 26.28 ± 2.13* | 0.016 | 44.70 ± 4.20* | 0.000 |
| Dose II          | 27.05 ± 7.17* | 0.013 | 46.82 ± 5.48* | 0.000 |
| Dose III         | 28.37 ± 7.09* | 0.010 | 52.92 ± 3.59* | 0.000 |

Description: Pre-prandial day 0-7* p < 0.05; significantly different with negative control  
Pre-prandial day 0-14* p < 0.05; significantly different with negative control

The results of the reduction rate of postprandial blood glucose levels (Table 3) on day 7 showed that the leaf extract of *A. macrocarpa* at doses of 12.5 mg/kg BW, 25 mg/kg BW, and 50 mg/kg BW had activity in lowering glucose levels.

### 3.3. Liver glycogen levels

Glycogen is the main source of polysaccharides in human and animal cells, which is a stored form of glucose found in almost all body tissues, especially in the liver and muscles. The purpose of glycogen stored by the body is as a temporary provider of glucose as an energy source. In DM condition, the body lacks of insulin or the body produces little insulin, so that the glucose that enters the cells is reduced resulting in a lack of glucose in the cells so that there is no possibility of accumulation of glycogen [19].

Liver glycogen levels of all test groups can be seen in the Table 4. Normal controls had the highest average liver glycogen levels. The negative control liver glycogen level had the lowest liver glycogen level because the rats were still in a hypoglycemic condition. The positive control group did not differ significantly with the dose group of 12.5 mg/kg BW (p = 0.564) and the dose 25 mg/kg BW (p = 0.083). This is in positive control rats given glibenclamide drug because the mechanism of glibenclamide drug can increase insulin secretion due to glucose stimulation [30]. The dose group of 12.5 mg/kg BW and 25 mg/kg BW could increase liver glycogen levels as well as positive controls given glibenclamide. The dose of 50 mg/kg BW (p = 0.083) was not significantly different from the normal control which showed the most significant increase in liver glycogen levels compared to the other two dose groups. Giving a
dose of 50 mg/kg BW in test animals was able to increase liver glycogen levels as well as test animals that were not induced by alloxan. This is because based on the results of the phytochemical compound test the n-butanol fraction of *A. microcarpa* leaves contains flavonoid compounds, tannins, and phenols which are thought to act as antidiabetic.

**Table 4. Liver glycogen levels.**

| Group            | Glycogen levels (µg/mg) | Mean±SEM |
|------------------|-------------------------|----------|
| Normal group     | 10.907±0.5723*          |          |
| Positive control | 2.061±0.3314            |          |
| Negative control | 5.3244±0.4670*          |          |
| Dose I           | 5.0086±0.3229*          |          |
| Dose II          | 6.5088±0.3215*          |          |
| Dose III         | 8.8513±0.4508*          |          |

Description: * p < 0.05; significantly different with negative control

The activity of reducing pre-prandial and postprandial blood glucose levels of the n-butanol fraction of *A. microcarpa* leaves was thought to be due to the presence of flavonoid compounds, tannins, and phenols. The methanol extract of the leaves of *A. microcarpa* with different species contains flavonoid and phenolic compounds [31]. Flavonoid compounds can counteract free radicals by donating hydrogen atoms to radical compounds to become more stable compounds, so that these flavonoid compounds degenerate pancreatic cells to produce more insulin which can reduce blood glucose levels [1]. Flavonoids can also reduce blood glucose levels by stimulating the release of insulin from cells that are not damaged [32]. Phenol compounds can increase insulin secretion and counteract free radicals in diabetic conditions [33]. This mechanism is similar to the positive control mechanism used, namely glibenclamide. Tannin compounds play a role in lowering blood glucose levels because tannins can induce phosphorylation of insulin receptors so that they stimulate the activity of glucose transporters, namely glucose transporter 4 (GLUT 4) which is found in cell membranes [34]. Some of these phytochemical compounds are also thought to increase liver glycogen levels. Flavonoid compounds play a significant role in increasing the activity of antioxidant enzymes, are able to regenerate damaged pancreatic cells, and can improve insulin receptor sensitivity. The ability of insulin to lower blood glucose concentrations by stimulating the use of glucose in muscle and fat tissue. There is an acceleration of blood glucose levels to the liver which is converted into glycogen, thus causing glycogen levels in the liver to increase [35]. Phenol compounds through the activation of glycogen enzyme synthesis can increase the process of glycogenesis, causing an increase in liver glycogen levels. Tannin compounds also have antioxidant activity and hypoglycemic activity that accelerate the entry of glucose from the blood into the liver and muscles, this is glucose converted into glycogen, causing glycogen levels to increase in the liver (increased glycogenesis) [36].

4. Conclusion

Based on the research and data analysis that has been done, it can be concluded that the n-butanol fraction of *A. microcarpa* leaves has activity in lowering blood glucose levels and can increase liver glycogen levels at all tested doses, namely doses of 12.5; 25; and 50 mg/kg body weight. Pancreatic histopathological test research needs to be done to determine the improvement in pancreatic cells.

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