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Protective Effect of Three Extracts of *Aquilariamicrocarpa* Baill against Alloxan-Induced Diabetic Rats with a Reference to Antioxidant Property.

Nurlely^{1*}, Noor Cahaya¹, Destria Indah Sari¹, Khoerul Anwar¹, Mustofa², Hidayaturrahmah³, Novieta Setiani Noor¹, Mega Permata Sari¹, Wenda Novita Sari¹, Ikrimah¹, and Aulia Rahmah¹

¹Pharmacy Department, Faculty of Mathematics and Natural Sciences, Universitas Lambung Mangkurat, Banjarbaru, South Kalimantan, Indonesia.

²Pharmacology and Toxicology Department, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

³Biology Department, Mathematics and Natural Sciences Faculty, Universitas Lambung Mangkurat, Banjarbaru, South Kalimantan, Indonesia.

ABSTRACT

Diabetes mellitus is a metabolic disorder which may be induced by oxidative stress exhibiting the beta cell destruction. The present study is to evaluate protective effect of three extracts (methanol, ethyl acetate and decoction) of *Aquilariamicrocarpa* Baill in order to scavenge free radical as well as decrease blood glucose level. Free radical scavenging activity was assayed by using the 2,2-diphenyl-1-picrylhydrazyl. Total flavonoids and phenolics contents was also investigated. Hypoglycemic potential was evaluated in alloxan induced diabetic rats for 14 days and measured by mainly estimating pre- and postprandial blood glucose and glycogen liver levels. All extracts significantly ($p < 0.05$) decreased pre- and postprandial blood glucose levels as well as increased glycogen liver level. Antioxidant study of three extracts revealed that they have antioxidant property. Methanol extract revealed the highest antioxidant capacity (IC_{50} value) as well as total phenol content while the highest total flavonoid content was ethyl acetate extract. Thus, from this study we can conclude that all extract possess antioxidant and hypoglycemic effects.

Keywords: *Aquilariamicrocarpa* Baill, alloxan, antioxidant

*Corresponding author

INTRODUCTION

Oxygen is the most fundamental element in this life which cells in our body use oxygen to generate energy. Free radicals are produced as a consequence of this process. They are reactive chemical entities which can be toxic in our body at high concentration resulting oxidative stress. Some degenerative diseases such as diabetes mellitus might be triggered by this oxidative stress [1]. Meanwhile, an antioxidant plays a protective role in controlling the continuous formation of these free radicals.

Uncontrolled and elevated free radicals could damage cell membrane of pancreatic β -cells triggering insulin deficiency and hyperglycemic condition. Diabetes is a metabolic disease characterized by the increased blood glucose level. This condition might be caused by the failure of the pancreas to produce enough insulin or the unresponsive cells to insulin that is produced or both [2,3].

Many medicinal plants have been used to treat diabetic patients to decrease their blood glucose level. Leaves of *Aquilariamicrocarpa* (*A. microcarpa*) Baill (local name : gaharu) have been empirically used by Dayak people in Central Kalimantan, Indonesia to treat diabetes by boiling them. Previous studies have evaluated the other species such as *Aquilariasp*, *Aquilariamalaccensis* for their activities to treat diabetes and as an antioxidant [4]. However, the antioxidant and hypoglycemic effect of *A. microcarpa* Baill from Central Kalimantan has not been carried out yet. Therefore, our present study was conducted to evaluate these activities using alloxan induced diabetic rats.

MATERIALS AND METHODS

Plant materials

Fresh samples of *A. microcarpa* Baill leaves have been collected from Tamiyang Layang, Central Kalimantan, Indonesia. The plant was taxonomically authenticated by Indonesian Institute of Sciences (LIPI), Bogor, Indonesia.

Sample preparation

Fresh leaves of *A. microcarpa* Baill were collected in the morning and then washed in tap water, chopped and shade dried. The dried leaves were then pulverized to fine particles using an electrical blender and passed through sieve No. 25. These samples were finally stored in an airtight container for further use [5].

Sample extractions

Each two hundreds grams of *A. microcarpa* Baill fine pulverized sample was extracted with methanol and ethyl acetate by percolation method. The mixtures were then filtered using Whatmann filter paper No. 1 and the extracts were evaporated using rotary evaporator at 50-55°C. The residue filtrates obtained were dried by waterbath at 50°C and stored in a refrigerator at 8°C for further experimental use. The yield values of the methanol and ethyl acetate extracts were 29 and 4.8% (w/w) respectively [6].

To obtain dosage of *A. microcarpa* Baill decoction of 100, 200 and 400 mg/kg BW; 2, 4, and 8 gram respectively of *A. microcarpa* Baill fine pulverized sample were weighed and boiled in 100 ml of water, allowed to stand for 30 min at temperature of 90°C and filtered through simple filter paper. The filtered sample was adjusted to 100 ml of water. A fresh decoction was prepared every 2 days and kept in a dark bottle at 8°C. [7].

Phytochemical screenings

Qualitative preliminary phytochemical screenings of methanol extract, ethyl acetate extracts and decoction of *A. microcarpa* Baill were determined according to the standard procedures to determine the presence of phytoconstituents like flavonoids, alkaloids, tannins and phenols [8,9].

Experimental animals

Healthy male Wistar rats (180-200 g) were purchased from UD Wistar, Yogyakarta, Indonesia. The animals were acclimatized for one week prior to the experiment in the standard laboratory animal room at 25±2°C with constant humidity (65%) and a 12 h light/dark cycle. The animals were fed with standard animal diet and water ad libitum during the experiment. The protocols of animals handling were approved by The Ethical Committee of Medical Research, Medical Faculty, UniversitasLambungMangkurat (No.007/KEPK-FK UNLAM/EC/III/2016).

DPPH free radical scavenging assay

Free radical scavenging assay was determined to evaluate the antioxidant capacity as described by Brand-Williams et al [10] with slight modification. Briefly, 1 ml of 0.4 mM DPPH solution in methanol was incubated with 4 ml of varying concentration of extract (4-100 ppm). The mixture was shaken well and incubated in the dark room at room temperature for 30 minutes. After incubation, the absorbance of each solution was measured at 515 nm using UV-VIS spectrophotometer. Different concentrations (1-5 ppm) of quercetin was used as a reference standard. Corresponding blank was prepared by adding vehicle to 1 ml of 0.4mM DPPH solution. Triplicate samples were used in this assay. The DPPH scavenging capacity of each sample was expressed as percentage of inhibition calculated by the following equation :

$$\text{DPPH scavenging capacity (\%)} = (\text{Ab}-\text{As})/\text{Ab} \times 100$$

Where, Ab = absorbance of the blank

As = absorbance of sample

Total phenolics content

The determination of total phenolics contents was measured using the method of Folin-Ciocalteu. Gallic acid was used as a reference standard with the concentration range: 20-100 ppm. The absorbance of each solution was measured at 754 nm using UV-VIS spectrophotometer. The total phenolics contents was expressed as gallic acid equivalents (GAE) in µg/mg of dry extract. Triplicate samples were used in this method [11].

Total flavonoids content

The total flavonoids content was determined by the aluminium chloride colorimetric assay. Quercetin was chosen as a reference standard with the concentration range: 60-140 ppm. The absorbance of reaction mixture was measured using UV-VIS spectrophotometer at maximum wave length of 416 nm. The total flavonoids content was expressed as Quercetin equivalent (QE) in mg/g of dry extract. Triplicate samples were used in this method [12].

Hypoglycemic effect

Animals were randomly divided into 12 groups of 4 rats each as follows :

Group 1 : normal rats received 0.5% NaCMC

Group 2 : diabetic rat

Group 3 : diabetic rats were administered glybenclamide 0.45 mg/kg BW

Group 4 : diabetic rats were administered methanol extract 50 mg/kg BW

Group 5 : diabetic rats were administered methanol extract 100 mg/kg BW

Group 6 : diabetic rats were administered methanol extract 200 mg/kg BW

Group 7 : diabetic rats were administered ethyl acetate extract 50 mg/kg BW

Group 8 : diabetic rats were administered ethyl acetate extract 100 mg/kg BW

Group 9 : diabetic rats were administered ethyl acetate extract 200 mg/kg BW

Group 10 : diabetic rats were administered decoction 100 mg/kg BW

Group 11 : diabetic rats were administered decoction 200 mg/kg BW

Group 12 : diabetic rats were administered decoction 400 mg/kg BW

All groups were fasted for 16 hours and then induced by alloxan (150 mg/kg BW) intraperitoneally. After 3 days of alloxan induction, preprandial blood glucose level was measured on day 3 after induction. Diabetic rats were determined if fasting blood glucose level > 200 mg/dl whereas, postprandial blood glucose level was measured 2 hours after glucose administration orally (1.75 g/kg BW).

All treatments were administered once daily after the determination of preprandial blood glucose level at observation day 0 until the next 14 days. After 14 days, pre- and postprandial were measured and liver organ were extracted out for the determination of glycogen liver.

The method of liver glycogen level was conducted by preparing 1 g of liver organ of rats and dried at 50°C for 1 night and then pulverized. Each sample was weighed 25 mg and extracted with 1 ml of 30% KOH and incubated in boiled water for 20 min. after that, 1.5 ml of 95% ethanol was added to the sample and kept at 4°C for 30 min and centrifugated at 2500 rpm for 20 min. Each sample was added with aquadest, 0/6 ml of 5% phenol and 3 ml of H₂SO₄ and measured the absorbance at 492 nm using UV-VIS spectrophotometer. Standard bovine glycogen liver was used to make calibration curve. All samples are in three replications. [13,14].

Statistical analysis

The data were statistically analyzed using the one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) test. Data were analyzed by SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis was set at the $p < 0.05$ and results were presented as mean \pm SEM.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

Preliminary phytochemical screening for different solvents of *A. microcarpa* Baill leaves revealed the presence flavonoids, tannins and phenols as showed in Table 1. The phytochemicals constituents were same for all extracts and decoction in the presence of flavonoids, phenols and tannins but absent for alkaloids.

Table 1: Phytochemical screening of methanol and ethyl acetate extracts and decoction of *A. microcarpa* Baill leaves

| Phytochemical Test | Methanol | Ethyl acetate | Decoction |
|---------------------------|-----------------|----------------------|------------------|
| Flavonoids | + | + | + |
| Alkaloids | - | - | - |
| Phenols | + | + | + |
| Tanins | + | + | + |

Total phenolics Content

The determination of total phenolics content of methanol, ethyl acetate and decoction of *A. microcarpa* Baill leaves used standard gallic acid for the extrapolation from the calibration curve as illustrated in Figure 1.

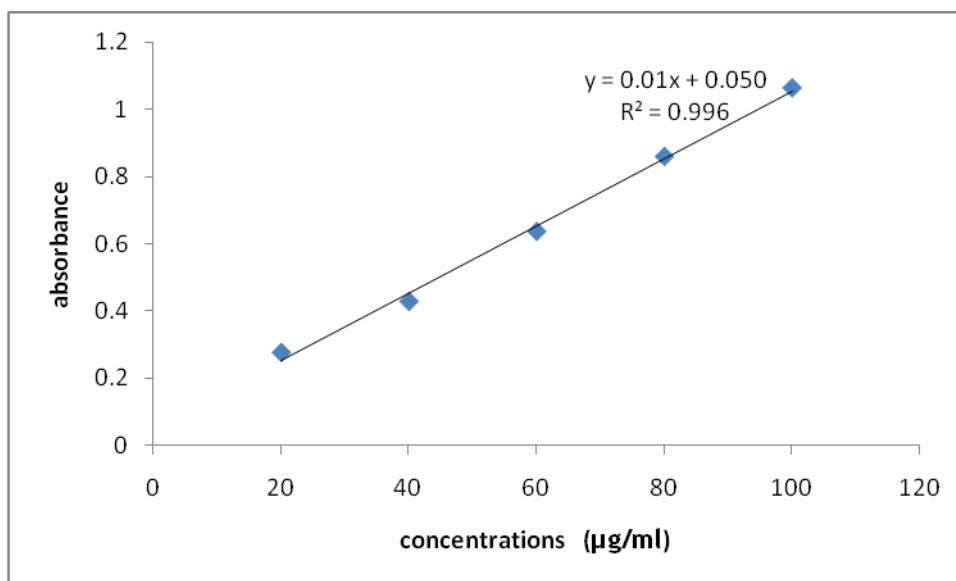


Figure 1: Calibration curve of standard gallic acid for determination of total phenols content

Total flavonoids phenols was determined by the extrapolation from the calibration curve of standard gallic acid. From table 2, it revealed that methanol extract contains the highest amount of total phenols content (534,43±0,47 mg/g) followed by ethyl acetate extract (62.92±0,46 mg/g) whereas decoction contains the poor amount of total phenols content (0.913±0,0047).

Table 2: Total phenols content of methanol, ethyl acetate extracts and decoction of the leaves of *A. microcarpa* Baill.

| Sample | Total phenols content* |
|-----------------------|------------------------|
| Methanol extract | 534,43±0,47 |
| Ethyl acetate extract | 62.92±0,46 |
| Decoction | 0.913±0,0047 |

*mg Gallic acid equivalent/g crude extract; Values are means of three biological replicates

Total flavonoids content

Standard quercetin (QE) was used to determine total flavonoids content of all samples of the leaves of *A. microcarpa* Baill as showed in calibration curve in Figure 2.

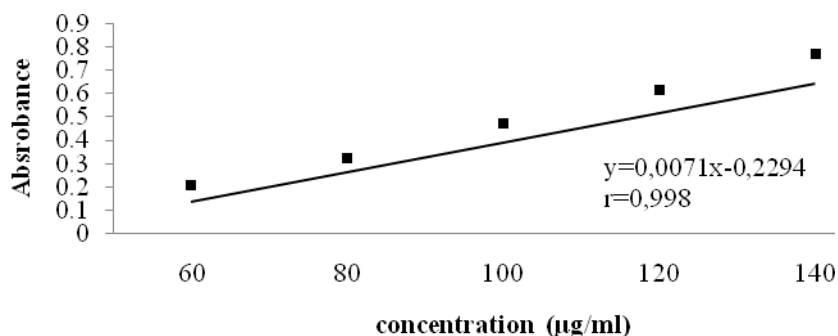


Figure 2: Calibration curve of standard quercetin for determination of total flavonoids content

Table 3 showed that ethyl acetate extract of *A. microcarpa* Baill contains the highest amount of total flavonoids content (475.08±3.40 mg/g) followed by methanol extract (117,24±0,50 mg/g) and decoction (82.38±0.50 mg/g) respectively.

Table 3: Total flavonoids content of methanol, ethyl acetate extracts and decoction of the leaves of *A. microcarpa* Baill.

| Sample | Total flavonoids content* |
|-----------------------|---------------------------|
| Methanol extract | 117,24±0,50 |
| Ethyl acetate extract | 475.08±3.40 |
| Decoction | 82.38±0.50 |

*mg Quercetin equivalent/g crude extract; Values are means of three biological replicates

DPPH free radical scavenging assay

From data in table 4, it revealed that methanol extract is the most effective of antioxidant followed by decoction and ethyl acetate extract.

Table 4: IC₅₀ value of some extracts / decoction and reference standard of quercetin

| Extract / decoction | IC ₅₀ ± SD (µg/ml) |
|---------------------|-------------------------------|
| Methanol | 15.59 ± 0.005 |
| Ethyl acetate | 58.31 ± 0.016 |
| Decoction | 24.52 ± 0.005 |
| Reference standard | |
| Quercetin | 3.68 ± 0.007 |

Hypoglycemic assay

Administration of methanol and ethyl acetate extracts and decoction of the leaves of *A. microcarpa* Baill for 14 days revealed the significantly decrease of preprandial and postprandial blood glucose level as showed in Figure 3 and 4 respectively.

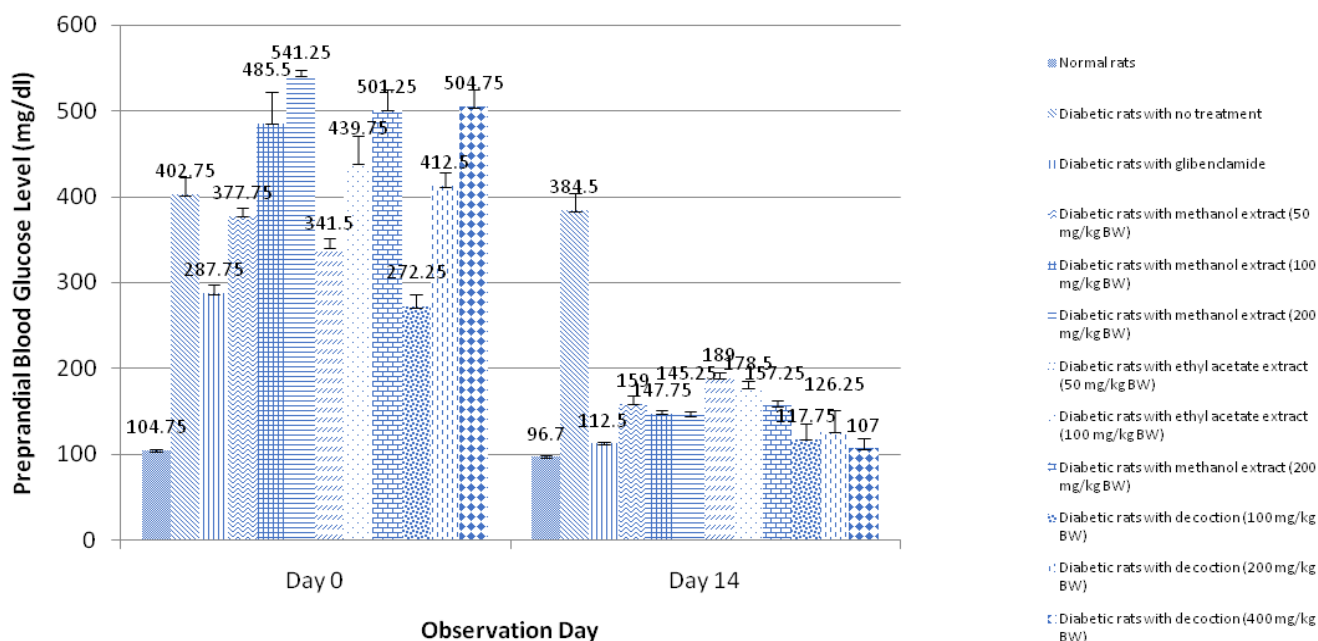


Figure 3: Preprandial blood glucose level on day 0 ad 14. each value represents Mean±SEM, n=4

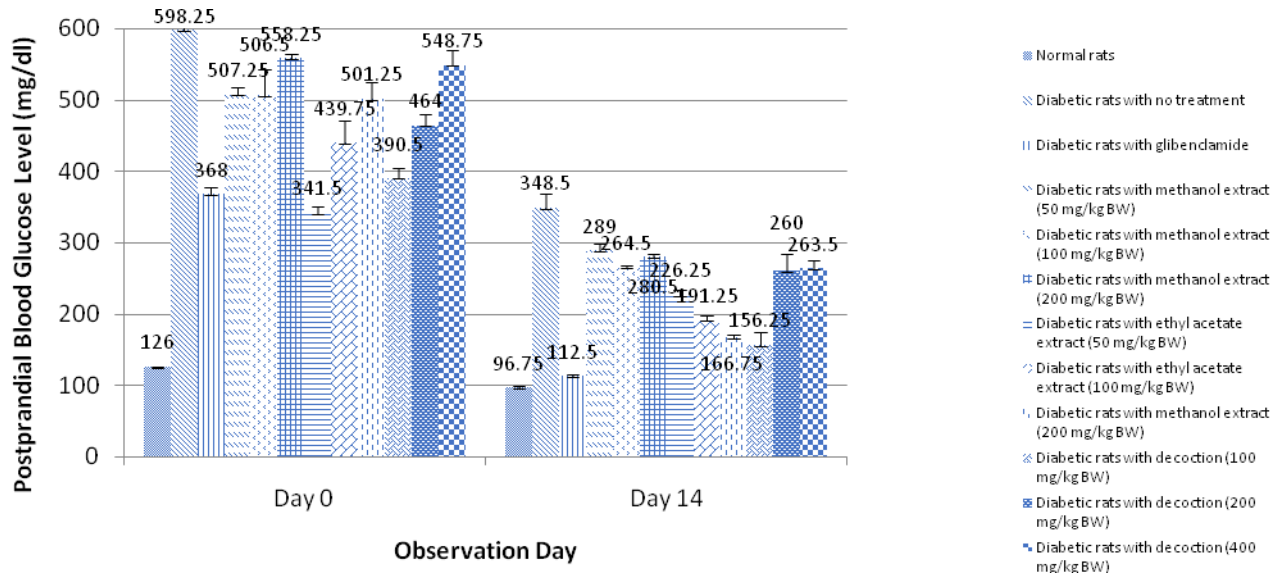


Figure 4: Postprandial blood glucose level on day 0 ad 14. each value represents Mean±SEM, n=4

After 14 days of administration of all extracts and decoction of *A. microcarpa* Baill, the percentage of pre- and postprandial glucose blood level were significantly decreased compared with diabetic rats with no treatment as showed in Figure 5.

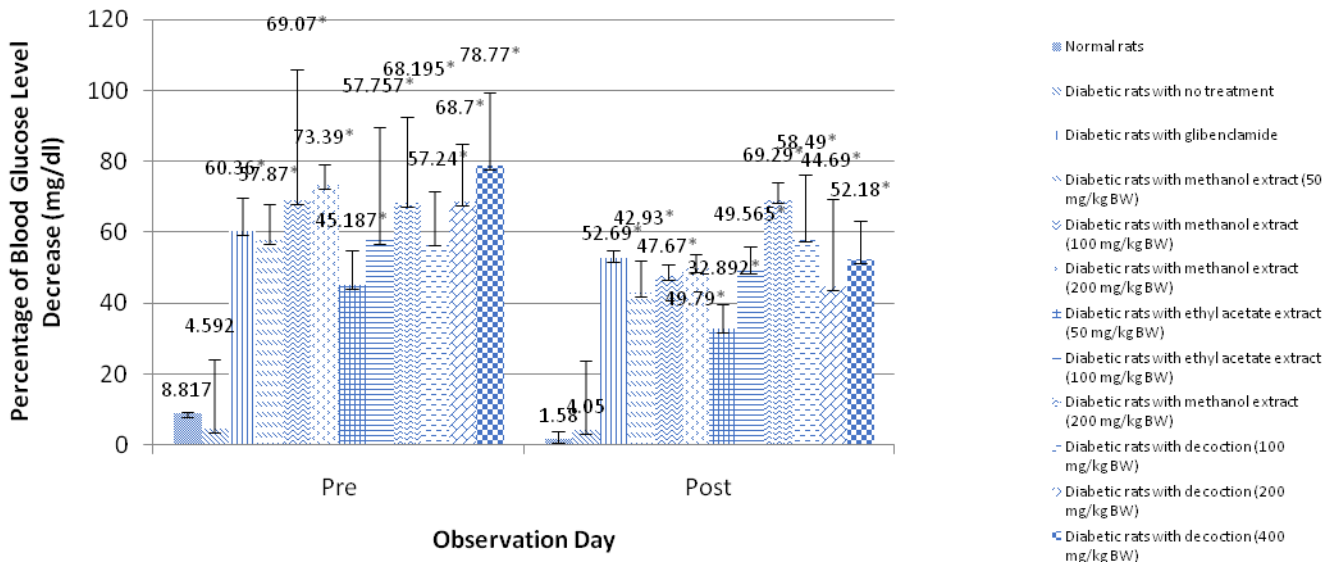


Figure 5: The percentage decrease of pre and postprandial blood glucose level * significantly decrease compared with diabetic rats with no treatment (P<0.05), each value represents Mean±SEM, n=4

Furthermore, the determination of liver glycogen level was measured using the extrapolation of calibration curve of standard bovine liver glycogen as illustrated in Figure 6. In addition, the administration of all samples of extracts and decoction of *A. microcarpa* Baill could significantly increased the liver glycogen level as showed in Figure 7.

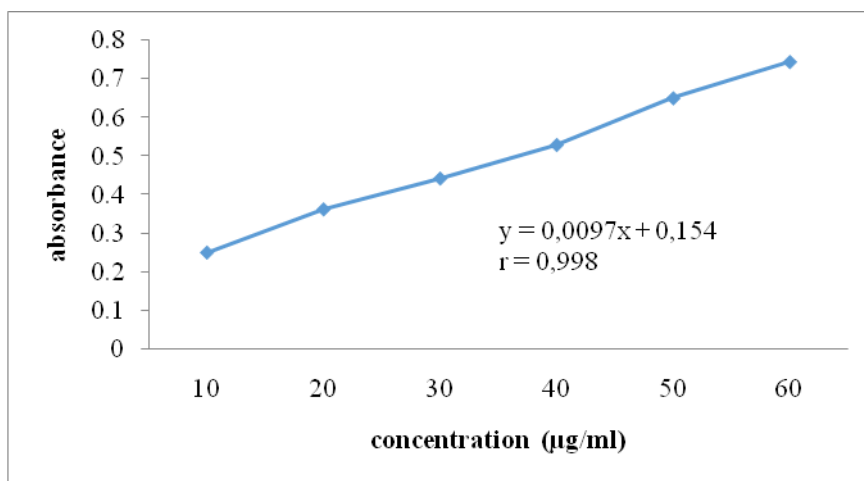


Figure 6: Calibration curve of standard bovine liver glycogen

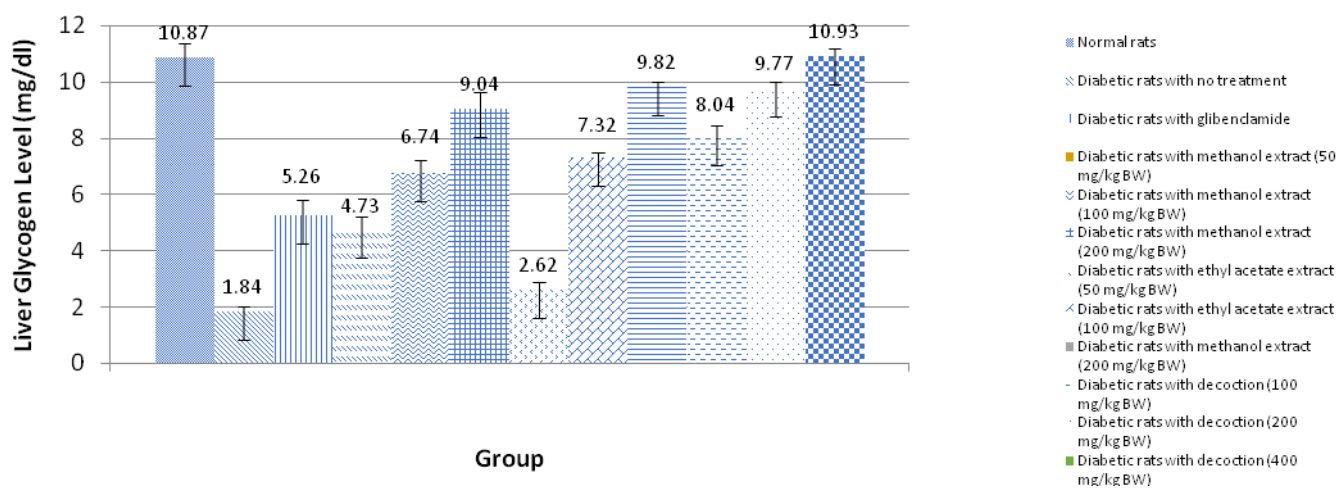


Figure 7: Liver glycogen level after 14 days treatment. * significantly decrease compared with diabetic rats with no treatment (P<0.05), each value represents Mean±SEM, n=4

DISCUSSION

Stem bark of *A. microcarpa* Baill possesses high economic utility and is well known as raw material in cosmeceutical industries. Other parts of this plant especially leaf, however, have been proven to treat many diseases such as diabetes mellitus, cancer and diarrhea. Based on the result of phytochemical screening, all samples of *A. microcarpa* Baill contain tannins, flavonoids and phenols. All these constituents have a contribution in scavenging free radicals [15].

Alloxan in this research is used as an inducer which has an ability to damage pancreatic β-cells. This chemical substance has a role as free radicals. When it is induced to the body, it converts to dialuric acid resulting reactive oxygen which can destroy pancreatic β-cells. This damage might cause the decrease of insulin production resulting hyperglycemic condition. [16].

Administration of all samples of *A. microcarpa* Baill for 14 days could decrease pre- and postprandial blood glucose levels. These effect are supported by the chemical compounds in samples such as flavonoids, phenols and tannins which can scavenge free radicals of diabetogenic effect of alloxan. Flavonoids have an ability to increase the action of GLUT 2 to increase insulin secretion as well as GLUT 4 translocation resulting the increase of glucose absorption into the tissue. Flavonoids also might decrease oxidative stress by donor hydrogen atom to free radicals generating more stabil substance and this effect inhibits the damage of

pancreatic β -cells. [17]. In addition, tannins could decrease blood glucose level by activating GLUT 4 mediators induced by insulin to strengthen the mechanism of glucose input. Tannins also could increase the activity of hexokinase which might increase insulin secretion [18]. Meanwhile, phenols could increase GLUT 4 as well as inhibit oxidative stress [19]

Flavonoids, tannins and phenols could also increase the liver glycogen level. Flavonoids might increase the level of liver glycogen by converting glucose to glycogen by increasing the secretion of insulin. Insulin could transfer glucose to produce energy. In addition, tannins accelerate the glucose input to the liver which can be converted into glycogen while phenols could increase glycogenesis process by glycogen synthase so could produce the increase liver glycogen [20].

CONCLUSION

In conclusion, methanol and ethyl acetate extracts and decoction from the leaves of *A. microcarpa* Baill could scavenge free radicals and decrease blood glucose level as well as increase glycogen level. However, it is necessary for further study to investigate their action mechanisms.

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