Antidiabetic Activity and Immunostimulant Potential of Bosibosi (*Timonius flavescens* (Jacq) Baker) Leaves Ethanol Extract in Alloxan-Induced Diabetic Rats

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Abstract. The number of people with diabetes mellitus (DM) is increasing alarmingly from year to year. Efforts to find cheap, safe and effective natural ingredients for DM are becoming increasingly important. This study was aimed to study the antidiabetic and immunostimulant properties of bosibosi (*Timonius flavescens*) leaves ethanol extract (LEE) in alloxan-induced diabetic female Wistar rats. Diabetic animals (2 mo, 150-190 g bw) were induced by ip administration of alloxan (175 mg/kg bw) after 24 hrs fasting. Animals were randomly distributed into 4 different groups (5 rats each), group I (normal rats, treated with NaCl), group II (diabetic rats, treated with NaCl), group III (diabetic, treated with metformin, 250 mg/kg bw), and group IV (diabetic rats, treated with LEE, 500 mg/kg bw). All ingredients (total volume 1 mL) were administered orally for 14 consecutive days. Blood samples were taken from the ventral tail vein on days 0, 7 and 14 for blood glucose determination. On day 14, rats were fasted (24 hrs), anesthetized and cardiac punctured for blood sample (5 mL) for leucocyte analysis. Blood glucose level of diabetic rats with EED (IV) was significantly lower than diabetic mice without LEE (II) (p <0.05). There was no significant difference in blood glucose level between LEE (IV) and normal (I) and metformin (III) rats. LEE also significantly increased the relative weight of lymph, the number of lymphocyte compared to normal (normal, non-diabetic) animals. Based on the results, it can be concluded that the ethanol extract of bosibosi leaves has antidiabetic activity and immunostimulant properties.

1. Introduction

The number of people with diabetes mellitus (DM) is increasing alarmingly. DM has become one of the main causes of death, both nationally and globally [1][2][3][4]. DM, which is characterized by high blood sugar level, is a disorder of carbohydrate metabolism caused by endocrine disorders. If not treated properly, this disease can lead to various complications [1][3][5][6][7][8][9] which results in premature death or a decreased in work productivity and life expectancy [10].
DM patients are generally treated with conventional pharmacological drugs, especially oral hypoglycemic drugs and insulin [11][12]. These drugs actually cause specific toxic side effects and can even reduce insulin receptor sensitivity and trigger insulin resistance [13][14]. In addition, these drugs have also become increasingly expensive, making it difficult for DM sufferers to reach, especially in low and middle income countries, such as Indonesia [15].

WHO has recommended the use of traditional medicines in health care, prevention and treatment of diseases (including DM), as well as encouraging efforts to increase the safety and efficacy of these medicines [16]. More and more people are using traditional natural medicines, especially those from plants, for the treatment of DM [17][18]. About 800 types of plants have been reported to have antidiabetic or immunostimulant potential [19], but only a small proportion of them have scientific basis.

Indonesia, as a megabiodiversity country, has approximately 40,000 plant species, of which 7,000 - 10,000 species have been used as medicinal plants for thousands of years [20][21][22]. One of the plants that has long been used by the community to lower blood sugar is *Timonius flavescens* (genus Rubiaceae) [23][24]). Other species of the genus Timonius have also been used traditionally as contraceptives and antidote [25], anti hypertension [26], anti malaria [27], lung disease and gonorrhea treatments, postpartum uterine cleansing and to inhibit bacterial growth [28]. Certain parts of the Timonius flavescens plant have been reported to have lipoxygenase inhibiting activity [29], anti-inflammatory [30] and inhibitor of muscarinic [31] and central nervous system receptors [32]. The use of traditional medicinal plants needs to be researched and developed so that they can be used by the wider community [33].

*Timonius flavescens* is widespread in the areas of Malesia, Mascarene, Malaysia peninsula, Indonesia (Borneo, Maluku, Papua and Sumatra), the Philippines, Australia, and the Tuamotu Islands [34][35][36]. In Sumatra this plant is found in Riau [37], Bangka [38], Jambi [39], and North Sumatra [40].

The purpose of this study was to explore the potential of the *Timonius flavescens* plant (local name bosibosi, in Tapanuli, North Sumatra) as a source of antidiabetic drugs and immunostimulant.

2. Materials and Methods

2.1. Animals and plants

Twenty female Wistar rats (2 - 3 months old, weight 150 - 190 g) were used in this study. Once obtained, the animals were acclimatized in the laboratory for 7 days (12 hours light / 12 hours dark, RT), and supplied with food (pellet 202C, Charoen Phokphand) and tap water *ad libitum.*

The leaves of bosibosi (*Timonius flavescens* (Jacq) Baker) were taken from plants that grow naturally on the hills of Siatas Barita, Simorangkir, Tarutung, Tapanuli Utara. Only dark green leaves, which were in the 3rd to 6th positions of the shoots, were used in this study. Fresh leaves were washed, drained and airdried in a room (25-30°C, humidity 55%) until the moisture content was ≤ 10% (moisturebalance). The dried leaves were then blended to form a powder (0.1 - 0.8 mm), put in an airtight plastic bag and stored in refrigerator (4°C) until extracted.

2.2. Extraction

Simplicia was macerated in 96% ethanol (ratio 1: 10, w/v) (24 hours, agitated every 5 hours) then filtered with Whatman No.1 paper, and repeated 3 times. The combined filtrate was then evaporated with a rotary evaporator to form a concentrated extract (crude extract), then stored in refrigerator (4°C) until used.

2.3. Induction of diabetes

The acclimatized animals were given freshly prepared alloxan monohydrate (dissolved in 0.09% NaCl, *ip*, 150 mg/kg bw). Two days after injection rats were fasted for 24 hours, then blood was drawn from tail ventral vein for blood glucose determination with a glucometer (Accu-Chek Performa, Roche Indonesia). Only animals with blood glucose levels above 200 mg/dL were categorized as diabetes and were included in the experiment.
2.4. Experimental design and treatment

The study was conducted following a randomized posttest control group design. Randomly, normal (non-diabetic) mice were assigned to one group (group I) and diabetic rats were distributed into 3 different groups (5 per group), groups II - IV. Group I and II were given only 0.09% NaCl, group III was given metformin (250 mg/kg bw) and group IV was given extract (500 mg/kg bw). Animals were treated daily for 14 days and all materials were given orally with a total volume of 1 mL.

2.5. Blood glucose

Blood glucose was measured by glucometer on day 0, day 7, and day 14 after fasting for 12 hours. The tail ventral vein of the rat was injured with a sterile lancet, the test strip was dipped in the blood that came out of the wound. Blood glucose levels (in mg/dL) can be read on a glucometer monitor.

2.6. Leucocytes analysis and spleen weight

On day 14, animals were anesthetized (diethyl ether) then a blood sample (5 mL) was taken by means of cardiac puncture for analysis of white blood cells.

The total number of white blood cells was counted with a blood sample of 0.5 mL inserted in a standard leukocyte pipette marked with “11”. Turck’s solution (to lyse erythrocytes) was added up to the "11" mark. The pipette was reversed for approximately 3 minutes so that the blood and reagents mixed well. The solution in the pipette was discarded as much as 2 or 3 drops. The rest was dropped in the counting chamber then left for 1 minute so that the cells were evenly distributed, and was examined under a microscope with weak magnification (10 times). White blood cells look like black patches. The total leukocytes in 4 large boxes were counted.

The type and number of white blood cells were determined using a smear technique. The blood sample was dropped on a clean slide surface and then fixed by adding methanol until the entire blood surface was well covered and then left for 6 minutes. Excess methanol was discarded then the preparation is dripped with Giemsa 10% solution in PBS (pH 7.4) and left (20 minutes). The preparation was then rinsed with distilled water and dried and then observed under a light microscope. Leukocytes totaling 100 cells were counted in different viewpoints and readings were made in the sections where the erythrocytes were close to each other but not overlapping or sparse. Each type of white blood cell was expressed as a percent (%) and the absolute number is calculated by multiplying the percentage of the number by the number of leukocytes, the results are expressed in cells/µL [41].

Spleen were taken and washed with PBS solution pH 7.4, cleaned of fat, then weighed with analytical scales. The relative weight of the spleen was determined by comparing it with the absolute body weight of the animals and expressed in percentage.

2.7. Statistical analysis

Results were expressed as mean ± SD for groups of five animals. Statistical differences in the means between the experimental groups were assessed using one-way analysis of variance and Tukey’s post hoc test. The means were considered statistically different at p < 0.05. All statistical analyses were performed using SPSS Statistics software v. 22.0 for Windows (IBM Corp., USA).

3. Results and Discussion

3.1. Results

3.1.1. Animal body weight. Body weight of all group of animals tended to increase during the treatment period, except for the diabetic rats group, there was a slight decrease. However, these changes in body weight were not statistically significant (Figure 1).
3.1.2. Blood glucose level. The antidiabetic activity of *bosibosi* leaf ethanol extract (LEE) was determined based on the fasting blood glucose levels of experimental animals (Table 1). The blood glucose of negative (normal, non-diabetic) and positive controls (diabetes) rats did not change during treatment. On the other hand, blood glucose levels of rats given metformin and LEE were both decreased with the same pattern on day 7 and day 14. Nevertheless, on day 14 the decrease in blood glucose of the LEE-treated rats never reached the normal blood sugar levels that could be observed in the metformin-treated one.

Table 1. Blood glucose levels (mean ± SD, mg/dL) in normal and alloxan-induced diabetic rats treated with *bosibosi* leaves ethanol extract (LEE).

| Groups                  | Blood glucose level (mean ± SD, mg/dL) at day ... |
|-------------------------|-----------------------------------------------|
|                          | 0                | 7                  | 14                   |
| Normal                  | 102.4 ± 5.4a     | 104.2 ± 5.5a       | 107 ± 12.1a          |
| Diabetes                | 389.4 ± 12.3b    | 370.2 ± 30.7b      | 397.2 ± 15.1b        |
| Diabetes + Metformin    | 386.6 ± 4.9b     | 206.2 ± 24.0c      | 118.6 ± 8.1a         |
| Diabetes + LEE          | 391.8 ± 9.7b     | 229.8 ± 20.4c      | 154.2 ± 15.7c        |

Different letter with the same column indicates significantly different (p < 0.05)

3.1.3. Spleen to body weight ratio. The relative weight of the spleen compared to the body weight of the rats at day 14 of experiment (expressed in %) is shown in Figure 2. It can be seen that the spleen relative weight of diabetic animals is significantly lower compared to normal and treated-diabetic animals, either with metformin or LEE. There was no significant difference in the relative weight of the spleen between normal rats and treated-diabetic rats given metformin and those given LEE.
3.1.4. Counts and type of leucocytes. The total number and type of rat leukocytes on the 14th day of the experiment are shown in Table 2. The data show that the total number of leucocytes of diabetic rats has decreased compared to normal and diabetic rats treated with metformin or LEE. There was no significant difference between normal rats compared with diabetic one given metformin or LEE. Diabetic animals treated with LEE experienced a very sharp increase in lymphocyte counts and a significant decrease in neutrophil counts.

Table 2. Total and type of leucocytes counts (mean ± SD) of normal and diabetic rats treated with LEE at the day 14 of the experiment.

| Leucocytes     | Normal     | Diabetes   | Diabetes+Metformin | Diabetes+LEE |
|----------------|------------|------------|--------------------|--------------|
| Total (10^3/mm^3) | 9.95 ± 0.72a | 8.67 ± 0.56b | 10.37 ± 0.93a | 9.99 ± 0.76a |
| Type and counts (%): |            |            |                    |              |
| Neutrophyll     | 64.68 ± 6.78a | 64.92 ± 7.76a | 63.00 ± 9.35a | 60.58 ± 6.29b |
| Eosinophyll     | 2.19 ± 0.28  | 2.22 ± 0.49  | 1.49 ± 0.18  | 1.25 ± 0.27  |
| Basophyll       | 0.82 ± 0.07  | 0.65 ± 0.09  | 0.78 ± 0.08  | 0.79 ± 0.08  |
| Lymphocyte      | 27.17 ± 0.66a | 26.69 ± 0.31a | 30.04 ± 0.42b | 33.16 ± 0.55c |
| Monocyte        | 5.13 ± 0.21  | 5.52 ± 0.19  | 4.69 ± 0.22  | 4.22 ± 0.17  |

Different letter between groups indicates significantly different (p < 0.05)

3.2. Discussion
In this study, increased blood glucose levels in alloxan-induced diabetic rats can be lowered to the level of metformin-treated diabetic or normal rats by administering the ethanol extract of bosibosi leaves (LEE). Hypoglycemic or antidiabetic activity of this extract may occur due to the potentiation of insulin from the islet β cells of Langerhans islet. The effect of lowering blood glucose is almost the same as that of metformin, a hypoglycemic drug. Until now, through qualitative identification, it is known that the ethanol extract of bosibosi leaves contains flavonoids, terpenoids and saponins [42]. There has been no quantitative research or elaboration on the types of these compounds in bosibosi leaves. However, it is widely known that various flavonoid compounds have antidiabetic activity in laboratory animals [43]. The antidiabetic mechanism of flavonoids can take various pathways, for example lowering blood
glucose, increasing insulin resistance and sensitivity, decreasing a-amylase and a-glucosidase enzymes, increasing pancreatic function, and others [43].

Organ weight is an excellent indicator of organ function. A number of factors have been reported that can affect organ weight of the animals, including strain, age, sex and environmental and experimental conditions [44]. This variation in body weight may be considered a potential source of bias in the analysis of organ weight data. Therefore, when considering organ weight data, the difference in body weight must be taken into account. The data of this study indicated that the spleen-to-weight ratio in diabetic control mice was lower than that in diabetic rats given alloxan or bosibosi leaf ethanol extract (LEE) and from normal control rats as well. The organ-to-body weight ratio may be associated with the occurrence of improvement and spleen function after the rats received metformin and LEE. As is known, the spleen is part of the lymph system or lymphatic system which functions to filter out damaged red blood cells and maintain and maintain the body's immune system. Cells in this system are known as immunocompetent cells, namely cells that are able to distinguish body cells from foreign cells or substances. The spleen is an organ that produces lymphocytes, so it is estimated that the heavier work of the spleen in producing lymphocytes can increase the size of the spleen [45].

Lymphocytes play a role in forming antibodies that protect the body against chronic infection and maintain certain immunity against infection. Bosibosi leaf extract may play a role in protecting the body from external factors, and providing immune defense through the induction of lymphocyte production. This can be seen in the increase of lymphocytes in rats given bosibosi extract through the number of lymphocytes found in normal control mice or diabetic rats given metformin (Table 2). One of the functions of lymphocytes is to produce antibodies. The resulting antibodies will carry out a specific immune response with antigens [46]. In addition, mice given the extract also showed an increase in the number of neutrophils on day 14. As is known, neutrophils function to prevent and fight bacteria invading the body [47]. All of these data, coupled with data on the increase in spleen-to-body weight ratio (Figure 2), indicate that the ethanol extract of bosibosi leaves has a good immunostimulant potential.

4. Conclusion

Based on the data obtained in this study, as discussed above, it can be concluded that the ethanol extract of bosibosi (Timonius flavescens (Jacq.) Baker) leaves has antidiabetic activity and potential as an immunostimulating agent.

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