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Antihyperglycemic Effectiveness Test of 96% Ethanol Extract from Soursop Leaves (Annona muricata L.) on Mus musculus Induced by Streptozotocin

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Abstract

Annona muricata L. is widely known throughout Indonesia as having great potentials as an antidiabetic agent. This study aimed to evaluate the antihyperglycemic effectiveness of ethanol extract 96% from soursop leaves and to compare its effects to metformin and insulin aspart injection as antidiabetic agents. This was a cross-sectional experimental study with random sampling approach which was performed at Phytochemistry and Biopharmacy Laboratory, Faculty of Pharmacy, Hasannudin University, Indonesia, from February 17 to March 3, 2020. Twenty-four Mus musculus subjects induced by streptozotocin were divided into six groups, which received 96% ethanol extract from Annona muricata in various doses. The extract was produced using the maceration process. The fasting blood sugar level was measured using the point of care testing (POCT) method, and data collected were then analyzed using a univariate approach. Results showed that the average fasting blood glucose level (FBG) was decreasing with increasing doses (5 g/kgBW=127 mg/dL, 10 g/kgBW =114 mg/dL, and 15 g/kg BW=97.75 mg/dl with a p-value of 0.000 based on repeated ANOVA. When compared to the positive control 1 (metformin), the decrease in FBG level in this control group (70.67 mg/dL) was better than in the group that received ethanol extract from Annona muricata L. In subjects in positive control 2 (insulin aspart), the average FBG level remained high, which was 473 mg/dL. Hence, 96% ethanol extract of Annona muricata L. can effectively lower fasting blood glucose in Mus musculus.

Key words: Antihyperglycemic, ethanol extract, soursop leaves (Annona muricata L.), streptozotocin

Uji Efektivitas Antihiperglikemik Ekstrak Ethanol 96% Daun Sirsak pada *Mus musculus* yang diinduksi dengan Streptozotocin

Abstrak

Tanaman sirsak menyebar di pelosok Indonesia serta banyak dimanfaatkan masyarakat sebagai obat herbal untuk berbagai macam penyakit, diantaranya sebagai antidiabetes. Tujuan penelitian ini adalah mengetahui efektivitas antihiperglikemik ekstrak ethanol 96% daun sirsak pada mencit yang diinduksi dengan streptozotocin dan membandingkan efeknya dengan metformin dan insulin aspart. Penelitian ini adalah penelitian eksperimental dengan desain potong lintang dan teknik sampling secara acak yang dilakukan di Laboratorium Fitokimia dan Biofarmasi Fakultas Farmasi Universitas Hasanuddin pada 17 Februari–3 Maret 2020. Sebanyak 24 ekor mencit yang diinduksi streptozotocin dibagi menjadi enam kelompok yang menerima ekstrak etanol 96% dari daun sirsak dalam berbagai dosis. Pembuatan ekstrak menggunakan proses maserasi dan untuk mendapatkan gula darah puasa (GDP) menggunakan metode *point of care testing* (POCT). Hasil penelitian menunjukkan bahwa rata-rata kadar GDP menurun dengan peningkatan dosis pada dosis 5 g/kgBB=127 mg/dL, 10 g/kgBB=114 mg/dL, 15 g/kgBB=97,75 mg/dL dengan nilai p=0,000 berdasar atas uji ANOVA. Jika dibanding dengan kontrol positif 1 (metformin) penurunan nilai GDP jauh lebih baik pada kelompok kontrol 70,67 mg/dL lebih baik dibandingkan pada kelompok yang mendapat ekstrak etanol dari daun sirsak. Pada subjek kontrol positif 2 (insulin aspart) nilai kadar GDP tetap tinggi, yaitu 473 mg/dL. Simpulan, ekstrak ethanol 96% daun sirsak efektif menurunkan kadar glukosa darah puasa pada mencit.

Kata kunci: Antihiperglikemik, daun sirsak, ekstrak ethanol, streptozotocin

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Introduction

Indonesia is widely known as a country with diverse plant species. Therefore, Indonesians are already familiar with various plants that can be used for traditional medicines as they are easy to find and affordable. One of such plants is the soursop leaf (*Annona muricata L).*[1]

Annona muricata L. contains elements of acetogenins, flavonoids, terpenoids, phytosterols, alkaloids, polyphenol, saponins, and tannins. These elements are essential as antitumor, antimicrobial, antiparasitic, and antiviral agents. This leaf is also known for its ability to cure skin diseases, rheumatism, cough, flu, anti-diabetic, and hypertension.[1] Compounds in soursop leaves that are considered to have anti-diabetic elements are alkaloid, flavonoid, and tannin.[1,2,3]

According to the ADA (American Diabetes Association) 2010, DM (diabetes mellitus) is a group of metabolic diseases indicated by hyperglycemia (high glucose levels in the blood) as the consequence of insulin secretion, insulin action, or both that leads to various chronic complications of the eyes, kidneys, nerves, heart, and blood vessels.[4]

In 2000, a report from WHO, based on the global death statistics, stated that 57 million annual deaths are caused by non-communicable diseases and that the WHO estimates an increase of 3.2 million of global deaths per year due to DM. Furthermore, WHO also estimated that 194 million people or 5.1% of 3.8 billion world population aged 20–79 years suffered from DM in 2003 from DM and will increase to 333 million by 2025.[5] The prevalence of type 2 DM in Caucasians ranges from 3% to 6% of their adult population. The frequency of diabetes also increases rapidly in the last ten years in Singapore. In the United States, the number of people with diabetes increases from 6,536,163 in 1990 to 20,676,427 in 2010.[6]

WHO predicted that Indonesia had experience an increase of 8.4 million of people with diabetes in 2000 and will see a rise to 21.3 million by 2030. This will position Indonesia on the 4th (fourth) rank globally after the US, China, and India in terms of diabetes prevalence.[5,7]

The acute metabolic complication and chronic vascular complications can lead to microangiopathy and macroangiopathy, as seen in uncontrolled diabetes mellitus (DM) cases. It includes, among others, neuropathy, nephropathy, retinopathy, coronary heart disease, stroke, and hypertension, which can be fatal.[7,8] With this in mind, this study aimed to evaluate the effectiveness of antihyperglycemic of 96% ethanol extract soursop leaves and compares its effects to metformin and insulin aspart injection as antidiabetic agents.

Methods

This was a cross-sectional experimental study conducted from February 17 to March 3, 2020 at the Laboratory of Phytochemistry and Biopharmaceutical of the Faculty of Pharmacy Faculty, Universitas Hasanuddin. The population used was white-squeaky males (*Mus musculus)* strain balb-c which were sampled using random sampling approach based on the inclusion criteria of males, aged 2–3 months, approximate weight 20–30 grams, having high FBG (>200 mg/dL) after injected with streptozotocin, and in healthy conditions. The minimum sample size (mice) for each group was four and the number of groups in this study was six, thus the minimum sample size was 24 mice. The mice were divided into the following groups: P1 (5g ethanol extract/kgbw), P2 (10g ethanol extract/kgbw), P3 (15g ethanol extract/kgbw), K +1 (metformin), K +2 (insulin aspart/novorapid), and K – (only regular food). The tools used were 1 cc syringe, gastric tube, analytical scale, blender, measure cup, Erlenmeyer glass, stirring stick, glass jar, porcelain cup, aluminum foil, sieve mesh 8, alcohol cotton, lancet, glucometer (Nesco multi check type N-01), glucose strips, gloves, mask, mortar and pestle, horn spoon, filter paper, rotary evaporator (Heidolph-VAP Core type HL/G3), water bath, and microwave. The materials used were PUR, streptozotocin, soursop leaves, metformin, insulin aspart (Novorapid), and 96% ethanol.

The extraction of A. Muricata L. was performed using maceration process by simple immersion with 96% ethanol in a glass jar for five days. The essence was collected by stirring and filtering the mixture at the same time. The resulting essence was then placed into the evaporator. Next, the solvent was separated and condensed using the water bath. In order to obtain the baseline Fasting Blood Glucose (FBG) level of the mice, capillary blood was sampled using the Point of Care Testing (POCT) method. Blood was collected from the tail after fasting for twelve hours by cutting the tip of the tail slightly. The glucose level was checked using a glucometer (Nesco).

Data used in this study were primary data consisting of mouse fasting blood glucose and

Figure 1 96% Ethanol extract from soursop leaves (A), STZ injection via intraperitoneal (B), oral administration of soursop leaf extract (C), subcutaneous administration of aspart insulin (D), weighing mice (E), fasting blood sugar checks (F)

weight before the intervention and after the streptozotocin induction and administration of 96% ethanol of soursop leaves/metformin/ insulin aspart. Data collected were then imported into Microsoft Excel 2013 and each variable scale was evaluated. Afterwards, a hypothesis test was performed using Repeated Anova to determine the difference between FBG levels before the intervention, after induction, and after extract administration. Furthermore, one way-Anova was also used to measure the difference in the FBG level decrease among the extract groups dose 1, dose 2, and dose 3, along with positive and negative controls. All statistical tests were performed using SPSS 25.

Results

After 16 days of treatment, a change in fasting blood sugar was observed (p=0.000) as shown in figure 2. The average FBG level in group P1 at the beginning of the study was 150 mg/dL, which increased to 430 mg/dL after streptozotocin administration. After 96% ethanol administration of 5g/kgBW, the rate decreased to 130 mg/dL. In Group P2 the average fasting blood sugar at the beginning of the examination was 150 mg/dL, which increased to 330 mg/ dL after streptozotocin administration and decreased to 110 mg/dL after the administration of 96% ethanol extract of 10g/kgBW. In Group P3, the FBG level increased to 280 mg/dL after streptozotocin administration and decreased to 150 mg/dL on average after the administration of ethanol extract. The positive control group 1 (K+1) indicated the average fasting blood sugar of 130 mg/dL, which escalated to 310 mg/dL by streptozotocin administration, and decreased to 70 mg/dL after metformin administration. The

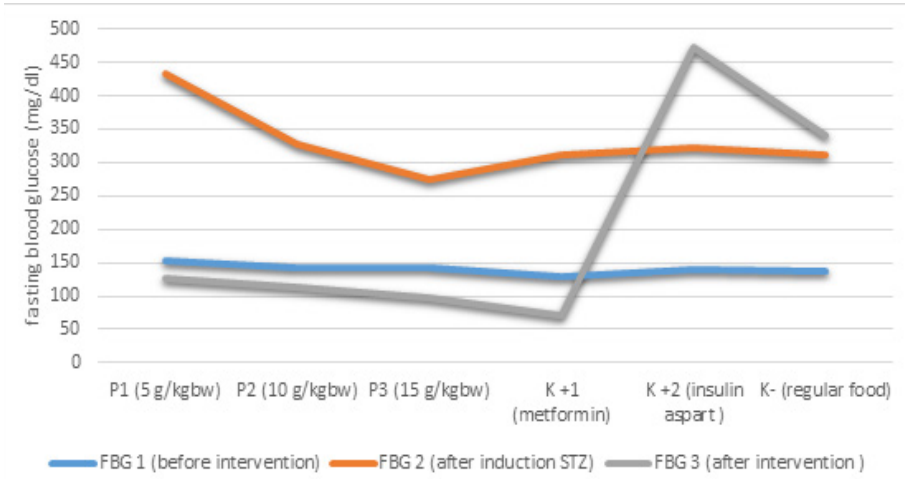

Figure 2 Average *Mus musculus* Fasting Blood Glucose Level in Three Measurement Times

average fasting blood sugar in the positive control group 2 (K+2) was 140 mg/dL. In contrast, the increased FBG level of 330 mg/dL was generated after streptozotocin administration, followed by a higher level of 480 mg/dL after insulin aspart administration. Although the negative control group (K-) started with an average 140 mg/dL fasting blood sugar, the normal food level remained high at 340 mg/dL and increased to 310 mg/dL (after STZ administration).

The change in weight was also observed after 16 days of treatment as described in figure 3. The average initial body weight in group P1 was 27.9 grams, then reduced to 26 grams (STZ administration), and 25.5 grams after the administration of 96% ethanol extract of soursop leaves of 5g/kgBW. Group P2 demonstrated an average initial body weight of 28.3 grams, which decreased to 27.1 grams after STZ administration and to 25.2 grams after the administration of 96% ethanol at 10g/kg BW. Similarly, the average initial body weight of 27.3 grams in group P3 also decreased to 26.3 grams after the STZ administration and 24.4 grams after the administration of 96% ethanol at a dose of 15g/kgBW. In the positive control group 1 (K+1) with average initial weight of 28 grams, weight reduction was also observed, down to 26.8 grams after streptozotocin injection and 25.2 grams after metformin injection. Meanwhile, the streptozotocin injection was seen to reduce the weight to 26.9 grams, and further injection of

Table Average Rate of Fasting Blood Glucose Decrease After Administration of 96% Ethanol Extract from A. Muricata L

Group	N	Mean of FBG	FBG min	FBG max
		(mg/dL)	(mg/dL)	(mg/dL)
P1 (5g/kgBW)	4	127,00	103	146
P2 (10g/kgBW)	4	114,00	106	127
P3 (15g/kgBW)	4	97,75	93	104
K +1 (metformin)	4	70,67	46	93
K +2 (insulin aspart/novorapid)	4	473,00	391	594
K – (only regular food)	4	341,50	289	419
Total	24	197,82	46	594

insulin aspart reduced the weight to 25 grams in the positive control group 2 (K+2), in which the average initial body weight was 27.9 grams. In the negative control group (K-) with an average initial body weight of 27.8 grams, a decrease to 25.2 grams after STZ administration and an increased to 26.5 grams by regular food (PUR) were observed.

Based on the result of the Repeated ANOVA statistical test, the P-value obtained is at the Greenhouse-Geisser=0.000. It showed significant differences at three different measurement times, i.e. before the intervention, after STZ induction, and after the administration of extracts and drugs.

In the follow-up ANOVA test to see the differences between the treatment groups and the control groups, a P-value of 0.000 was obtained. Since the P-value was <0.05, it can be said that 96% ethanol extract of soursop leaves was effective for reducing blood sugar level in mouse diabetic model.

Based on the research results, it was found that there was a significant decrease in GDP after giving 96% ethanol extract of soursop leaves compared to before giving the extract. Then from the 3 doses of extracts, including 5 g/kgBW, 10 g/kgBW and 15 g/kgBW, the most decreased was the P3 group with a dose of 15 g/kgBW (range 97.75 mg/dL), then P2 with a dose of 10 g/kgBW (range 114 mg/dL), then the P1 group with a dose of 5 g/kgBW (range 127 mg/dL). However, when compared with positive control 1 (metformin group), the metformin group had a better reduction compared to ethanol extract 96% of soursop leaves (range 70.67 mg/dL) and when compared with positive control 2 (insulin aspart group), the decrease of soursop leaves was better, which was the positive control value 2 (range 473 mg/dL), and when compared to the negative control (regular feed group), the soursop leaf extract had the better decrease, which was the negative control value (range 341, 5 mg/dL).

The ability of soursop leaves to lower blood glucose is due to the active substances they contain, including alkaloids, flavonoids and tannins.

Discussion

Streptozotocin (STZ,2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is a chemical compound synthesized from *Streptomycetes achromogenes* and used to induce both type 1 and type 2 diabetes mellitus. Streptozotocin enters β cells through the glucose transporter (GLUT 2) and causes DNA alkylation. DNA damage induces poly ADP-ribosylation activation, leading to depletion of cellular NAD + and ATP. An increase of ATP dephosphorylation after streptozotocin induction produces substrates for xanthine oxidase catalytic reactions. Further, it generates superoxide radicals and, as a result, the formation of hydrogen peroxide and hydroxyl radicals happens. Afterwards, streptozotocin frees many toxic substances from nitric oxide, which inhibits aconitase activities, and plays a role in DNA damage that eventually results in apoptosis and necrosis of β cells. Mice given

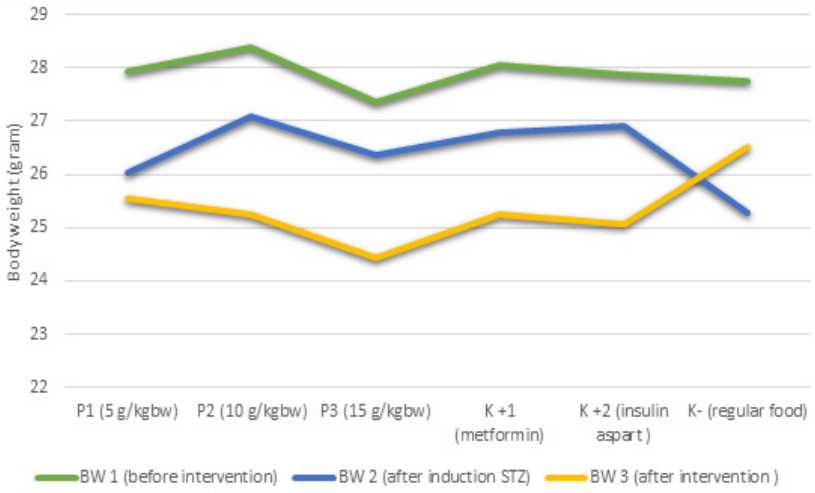

Figure 3 Mean *Mus musculus* Body Weight in Three Measurement Times

STZ experience apoptosis and beta-cell necrosis. Thus, STZ has an impact on insulin production and its functions.[9,10]

Based on the results of this study, a significant reduction in FBG is seen after the administration of 96% ethanol extract soursop leaves when compared to before the extract administration. Soursop leaves contain many useful elements, including flavonoids, alkaloids, and tannins, which are anti-hyperglycemic in nature.[1,2,3] Flavonoids play the role of antioxidants and can ultimately deduct insulin resistance. Other mechanisms, specifically those involving quercetin flavonoids, can inhibit GLUT 2 in the intestinal mucosa, which can reduce the absorption of glucose and fructose in the digestive tract. Thus, the blood glucose level can eventually decrease. [11]

Alkaloids have implications as a primary component in some natural plants that has an antidiabetic property. It has the ability to repair the GLUT 4, which functions to regulate insulin, reserves glucose storage in adipose tissue, and the striatal muscle (skeletal muscle and heart muscle). Also, it improves glucokinase activities, or liver enzyme activities, in regulating glucose level in the blood, and repair PPARγ (*Peroxisome Proliferator Activator Receptor*), which is a transcription factor. Alkaloids attach to the nucleus membrane, which has a potent anti-inflammatory effect, reduces glucose-6-phosphatase activity, and improves liver glycogen. In addition, a decrease in the activity of phosphoenolpyruvate carboxylase and aldose reductase will influence the glycolysis.[12]

Tannin acid stimulates glucose transport and inhibits differentiation in 3T3-L1 adipocytes by preventing genes of adipogenesis. It also deters the activity of α-amylase, which is a useful enzyme to break down complex carbohydrates into simple sugars. Furthermore, it inhibits α-glucosidase, an enzyme degrading glucose, to be absorbed and enter the bloodstream.[12]

This study uses metformin as the positive control 1. Metformin is an oral antihyperglycemic drug, which is the drug of choice for people with type 2 diabetes, that is considered to have an excellent working mechanism in suppressing glucose production in the liver, thus inhibits gluconeogenesis, and increasing insulin sensitivity. [13,14,15] This is in line with the result of this study, which demonstrates the effect of metformin in reducing blood sugar in experimental animals, which is stronger than the effect of ethanol extract of 96% soursop leaves.

Oral antihyperglycemic drugs are classified based on their function targets into those that increases insulin secretion, those that suppresses liver glucose production and increases insulin sensitivity, those that inhibits glucose absorption, those that increases insulin sensitivity only, those that increases insulin sensitivity and inhibits glucagon secretion, and those that inhibits glucose reabsorption in renal distal tube.[14] Metformin was selected as the drug used in the positive control due to the fact that it is a standard drug in the primary care due to its minimum side effect. Metformin is mostly prescribed for people with type 2 diabetes. It works by suppressing hepatic glucose production or inhibiting gluconeogenesis and increasing insulin sensitivity. Similarly, the mechanism of action of the active substance in A. Muricata L. as an antidiabetic, i.e. flavonoids, is by activating insulin receptors that has antioxidant potential for beta-cell repair and inhibiting glucose absorption through GLUT-2 transporter inhibition. Alkaloids also repair glycogen in the liver, reduce glucose-6-phosphatase activity, improve GLUT-4, glucokinase and PPAR activities. and decrease the activity of phosphoenolpyruvate carboxylase and aldose reductase. Lastly, tannins stimulate glucose transport and inhibit adipogenesis gene, α activity-amylase, and α-glucosidase.[11,12-15]

Another drug used as control in this study is insulin aspart. However, the results showed the absence of effect towards the hyperglycemic conditions, which can be caused by improper use. There are several indications for insulin use including HbA1c >9% with metabolic decompensation. Other conditions that become the indications for insulin use are rapid weight loss, severe hyperglycemia associated with ketosis, hyperglycemia crisis, optimal combination failure of oral antihyperglycemic drugs, and severe stress (systemic infection, major surgery, acute myocardial infarction, and acute stroke). In addition, it is also used for pregnancy with gestational diabetes or diabetes mellitus without meal planning control, which may lead to severe impairment of kidney or liver function.[13,15] Insulin is a proper diabetes therapy to reach blood sugar targets and is a preferred method compared to other methods. Earlier use of insulin is assumed to improve pancreatic β cell production and prevent the use of some drugs or insulin combination in the future.[2] Ideally, insulin is given according to the physiological state of the body and insulin therapy is provided once as basal insulin and three times as prandial insulin after meals.[7,8]

It can be seen in all groups, that the average weight loss occurred after hyperglycemic

induction using STZ. After three days of extracts and drug interventions in groups P1, P2, P3, K+1, and K+2, a reduction was observed after three intervention days while an increase in weight was seen in group K-. The hyperglycemia condition in type 2 DM disease and STZ induction results in a pancreatic beta-cell damage known to produce insulin hormone function. In both conditions, glucose that enters the body cannot be converted into glycogen and stored in the liver or muscles. Thus, weight loss occurs.

Bodyweight was measured to see effect of induction and administration of soursop leaf extract on body weight. Since DM tends to lead to weight loss, it is expected that after the administration of soursop leaf extract led, an increase in body weight will be observed, along with a decrease in blood sugar values. However, the results in our study fails to show an increase in weight.

Soursop extract elements that are expected to be anti-diabetic in nature include flavonoids, alkaloids, and tannins, where the compounds affect weight loss through the mechanism of adipogenesis gene inhibition and repair of GLUT-4 in adipose tissue and skeletal muscle.[14] Therefore, this study supports finding from other studies that persistent weight loss is seen in mice despite ethanol extract administration.

Metformin is the drug choice for overweight patients who fails a strict diet to control diabetes. When appropriate, it can also function as an alternative therapy for patients with average weight. Metformin is a hyperglycemic agent that increases glucose tolerance in type 2 DM patients and decreases basal blood sugar and post-prandial blood sugar. The pharmacological mechanism of this drug differs from other classes of antihyperglycemic drugs. It reduces glucose production in the liver, decreases intestinal glucose absorption, and increases insulin sensitivity by rising peripheral glucose.[8] In the negative control (K-), the body weight increases after three days of regular feeding. It means that under natural conditions, body weight can increase only by consuming regular food, without any other intake.

This study has limitations in that the results in control group 2 (insulin) is not as expected due to improper use that may involve inappropriate indications of administration and the necessity to combine prandial insulin with basal insulin to work more effectively in reducing fasting blood sugar. In conclusion, 96% ethanol extract of soursop leaves *(Annona Muricata L.)* significantly reduces the fasting blood sugar in *Mus musculus* when compared to metformin. This means that the soursop leaf extract has a prominent effect in decreasing fasting blood glucose.

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