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Original Article

ROSELLE CALYX (*HIBISCUS SABDARIFFA*. L) AS AN ANTI-DIABETIC: ETHYL ACETATE FRACTION REDUCE FASTING BLOOD GLUCOSE TOTAL CHOLESTEROL AND REPAIR PANCREAS FUNCTION ON DIABETIC MODEL

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ABSTRACT

Objective: In general, people with diabetes mellitus will experience disturbances in fat metabolism that lead to hypercholesterolemia. This study aims to determine the effect of ethyl acetate fraction hibiscus calyx on blood sugar, blood cholesterol level, and pancreas histology in diabetic Wistar Kyoto rats induced by streptozotocin.

Methods: Thirty-six Wistar Kyoto rats were induced with intra-peritoneal streptozotocin at 55 mg/kg BW and stabilized for five days to obtain diabetic conditions. Diabetic animals were divided into four groups; the diabetic group was given vehicle, the glibenclamide group was given 0.45 mg/kg BW of Glibenclamide, and two groups were administered the ethyl acetate fraction of hibiscus calyxes (EAFHC) at doses of 100 mg/kg BW and 200 mg/kg BW for five days. Fasting blood sugar and lipids (total cholesterol and triglycerides) were measured on days 0, 1, 3, and 5. Pancreats were collected on days 1, 3 and 5 for weighing and histology examination. All data were analyzed using two-way ANOVA followed by the Duncan Multiple Rank Test (DMRT).

Results: EAFHC significantly reduced fasting blood sugar and total cholesterol ($p<0.05$) but did not have a significant effect on triglycerides ($p>0.05$). Histology examination showed that EAFHC repaired pancreatic damage, as seen from the decrease in pancreatic histology scores ($p<0.05$), but the organ ratio did not show a significant improvement ($p>0.1$).

Conclusion: This study revealed that EAFHC could be an alternative medicine in managing blood sugar levels and total cholesterol and improving pancreas function in associated models of diabetes mellitus hypercholesterolemia complications.

Keywords: Antioxidant, Blood glucose, Hibiscus sabdariffa, Total cholesterol, Triglycerides, Histopathology

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder with high blood glucose levels. Insulin helps to maintain blood glucose levels by increasing glucose absorption in cells and suppressing hepatic gluconeogenesis. The International Diabetes Federation (IDF) reports that people with diabetes globally at 20-79 y in 2021 are estimated at 536.6 million people, increasing to 783.2 million people in 2045. Indonesia will occupy fifth place for diabetes in adults by 2021 and 2045 [1]. In general, 90% of the prevalence of diabetes mellitus is type 2, caused by an unhealthy lifestyle [2].

Organs directly related to the pathophysiology of diabetes is the pancreas, which consists of exocrine and endocrine tissues. The endocrine pancreas arranged on the Langerhans islands has four types of cells: alpha cells, beta cells, delta cells and cells polypeptide pancreas, respectively producing hormone glucagon, insulin, somatostatin and polypeptides pancreas. All hormones participate in the arrangement rate of glucose blood [3]. Insulitis tends to develop in diabetes mellitus patients. This syndrome is characterised by inflammation of the pancreatic islets of Langerhans, resulting in decreased insulin secretion [4]. The beta cells of the islets of Langerhans will suffer structural damage, deterioration, degeneration, and mass loss in this disease [5].

In general, diabetes mellitus patients will experience lipid metabolism disorders, which are distinguished by increased cholesterol levels or hypercholesterolemia [6]. Hyperglycemia conditions in diabetes mellitus can increase glucose autooxidation to form reactive oxygen species (ROS). Uncontrolled ROS production will cause oxidative stress conditions, namely an imbalance between ROS and the antioxidant defence system in the body and various other substrates such as amino acids and lipids [7].

In insulin resistance, the oxidation profile of atherogenic lipoproteins, particularly small dense LDL-cholesterol, increases due to increased activity of lipid cells' hormone-sensitive lipase and lipoprotein lipase. Hormone-sensitive lipase activity causes increased triglyceride lipolysis in adipose tissue. This process produces excess free fatty acids carried into the bloodstream. Some of these free fatty acids are used as an energy source, and others are used as raw material for the formation of triglycerides in the liver. In the liver, excess free fatty acids stimulate the conversion of phospholipids and cholesterol, which will be released into the blood in the form of lipoproteins. It can cause an increase in total cholesterol in diabetes mellitus patients [8–10].

The normal amount for total cholesterol is 200 mg/dl, according to the National Cholesterol Education Programme, Adult Panel Treatment III (NCEP ATP III) [11]. Cholesterol levels can be reduced by pharmacological and non-pharmacological treatment. The statin class of drugs is frequently used to decrease cholesterol levels. Statins are predicted to lower LDL cholesterol, raise HDL cholesterol, and lower triglycerides without causing significant adverse effects [12].

Nowadays, many people choose to use traditional medicine to treat health problems since the adverse effects are said to be less severe and the cost is cheaper than that of contemporary medication. Hibiscus sabdariffa. L (HSL), also known as red sorrel or roselle, belongs to the Malvaceae family and has been used as a medicinal herb in Africa, Asia, and Latin America to treat hypertension, urinary bladder, and kidney stones, as a diuretic, and to treat liver problems [12]. The antioxidant properties of HSL calyxes, shown by gossypetin, anthocyanins, hibiscus glycosides, and other flavonoid

compounds, provide noticeable health benefits [13]. Anthocyanin is an antioxidant that can lower blood glucose by increasing insulin and glucosidase sensitivity in the intestinal lumen.

Meanwhile, *in vitro* studies show anthocyanins can stimulate insulin release [14]. Rosella contains 33.9% soluble compounds that can help to remove fat. Anthocyanins also can reduce blood pressure by preventing atherosclerosis and lipid oxidatio. The research by Sapian *et al.*, 2023 revealed the effect of roselle calyx in regenerating the damaged-pancreas cells against diabetic condition [15].

Based on the information above, this study aims to determine the effect of ethyl acetate fraction hibiscus calyx on blood sugar, blood cholesterol level, and pancreas histology in diabetic Wistar Kyoto rats induced by streptozotocin. Until today, there is no available information regarding the effect of various doses and durations of ethyl acetate fraction of hibiscus calyxes on blood cholesterol, bleeding time, and pancreas histology analysis in rats with diabetes mellitus. With this study, data on the effectiveness of the ethyl acetate fraction of hibiscus calyxes as an anti-diabetic agent can be obtained, which could support the development of medication containing hibiscus.

MATERIALS AND METHODS

Plant material

Hibiscus calyxes were obtained from Kapalo Hilalang, Kayu Tanam area, Padang Pariaman Regency, West Sumatra. The calyxes were harvested directly from the stems of the hibiscus plant. Plant identification was conducted at the ANDA Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang.

Chemical material

Streptozotocin and Glibenclamide provided by Merck (Germany). Assay kits used for lipid profile (total cholesterol, triglyceride, and lipoproteins were obtained from Sigma–Aldrich (St. Louis, USA). Hematoxylin, Eosin dyes, formalin buffer, ethyl acetate, xylol and other chemicals used are analytical grade and obtained from commercial source.

Hibiscus calyx extraction and fractionation procedure

Fresh HSL calyx was cleaned and dried at room temperature for five days. The calyx was then pulverised to a mesh size of 60 and extracted with 70% ethanol for 18 h at a ratio of 1:5. The mixture was filtered using filter paper, then the resulting extract was filtered and concentrated using a rotavapor; the process was repeated twice to achieve complete extraction. After that, 40.13 gram of HSL crude extract were dissolved in a water-ethanol (9:1), in 150 ml solvent. the crude extract was fractionated by liquid-liquid extraction with hexane and ethyl acetate in increasing polarity order. Subsequently, the partitioning process was carried out using a separating funnel. The fractionation process ended when the n-hexane and ethyl acetate solvents, each forming a distinct upper layer and, became clear. The obtained ethyl acetate fraction was then collected. Finally, the fractions' solvent was removed using a rotary evaporator and desiccator [16].

Physical and physicochemical analysis

The physical and physicochemical properties, such as moisture content, volatile oil, and ash levels, were assessed according to the Indonesian Herbal Pharmacopoeia standard. The data was collected and given as the mean standard deviation.

Experimental animals

Wistar Kyoto rats weighing 180-200 g were used in this research. Animals obtained from animal house Faculty of Pharmacy, Universitas Andalas, Padang, West Sumatra, Indonesia. Animals are kept individually in stainless steel cage below cycle light and dark 12 h (24±2 °C and humidity relatively 40-60%). Water and food provided ad libitum. All test carried out in accordance with the Guidelines National Institute of Health for care and use of laboratory animals and the European Council Directive of 24 November 1986 in Care and Use of Laboratory Animals (86/609/EEC), and approved by the Research and Ethics Committee of the Faculty of Pharmacy, Andalas University (No. 32/UN. 1610. D./KEPK-FF/2023).

Diabetes induction

Streptozotocin at a dose of 55 mg/kg BW was injected intraperitoneal. Before streptozotocin induction, the rats were fasted for 16 h, to avoid hypoglycemia; the animals were given a 10% glucose solution and food for 24 h and continued with regular drinking water the following day. On the fifth day after streptozotocin induction, the blood glucose levels of the test animals were measured using Easy Touch ® GCU. The consistency and evaluation between the blood glucose levels measured by glucometers and plasma glucose levels measured by a laboratory biochemical test in rats have been done in our previous research [17].

Experimental design

Thirty-six diabetic rats were divided into four groups of nine rats each randomly. Group I, as the diabetic control, was given a vehicle (5% Na CMC). Group II was given glibenclamide at 0.45 mg/kg BW as a positive control (Glibenclamide). Groups III, IV, and V were treated with EAFHC at 100 and 200 mg/kg BW once daily for five days.

Blood glucose measurement

On days 1, 3, and 5, fasting blood sugar levels were assessed. An Accu-check active glucometer (Roche, Germany) was used to test blood glucose levels in the rat tail. The previous study's equation was applied to compute the percentage of fasting blood glucose change [17, 18].

Lipid profile measurement

On day 0, 1, 3 and 5 the blood was taken from the ocular sinus and centrifuged at 3000 rpm for 10 min, with the plasma remained. Total cholesterol (TC) and triglyceride (TG), levels in plasma were analyzed according to manufacturer procedures (Sigma-Aldrich, St. Louis, USA) [19].

Measurement results of lipid profile is presented as percent changes:

$$\% \text{ Lipid profil change} = \frac{\text{lipid profil 0} - \text{lipid profil 1}}{\text{[lipid profil 0]}} \text{x } 100\%$$

Pancreatic organ ratio measurement

Pancreatic organs taken on days 1, 3 and 5. Then, the organs are weighed and counted pancreatic organ ratio by:

$$\text{Pancreatic Ratio} = \frac{\text{Pancreatic Weight (g)}}{\text{[Body Weight (g)]}}$$

Histology examination of the pancreas

Before being cut, the pancreas was fixed in a 10% buffered formalin solution for 24 h. The pancreas tissue was dehydrated in 70% alcohol, 80% alcohol, 90% alcohol, 96% alcohol, xylol, and liquid paraffin in that order. The following steps are tissue vacuuming and embedding. The pancreas tissue was sectioned and stained with hematoxylin and eosin at a thickness of 4-6 mm. A 40 times magnification microscope with Optilab® was used to examine pancreas tissue in five distinct fields of view. ImageJ software calculated the stained area and staining intensity (NIH-Bethesda, MD, USA). Each biological copy was photographed separately. The five fields' average stained area and staining intensity were chosen as a sample of that duplicate. A semi-quantitative histopathology score modified from the recently acknowledged AASLD criteria was used to assess the histopathological characteristics of pancreas damage [20, 21].

Table 1: Physical and physicochemical parameters of H. sabdariffa calyx

No	Parameters	Values (in percentage)
1	Moisture content	7.82±0.43
2	Volatile oil	Nil
3	Total ash	3.18±0.11
4	Acid insoluble ash	0.93±0.51

Data analysis

The data was analyzed using two-way ANOVA, then followed by the Duncan Multiple Range Test (DMRT). $p<0.05$ were labeled statistically significant.

RESULTS

Physicochemical and phytochemical analysis

Table 1 shows the physical and physicochemical features of *H. sabdariffa* calyx. All parameter were according to Indonesian Pharmacopea standard.

EAFHC effect on blood glucose

The average drop in blood glucose levels for the diabetic control, glibenclamide group, EAFHC 100, and EAFHC 200 units, fig. 1. The administration of the EAFHC had a significant impact on blood glucose change, according to one-way ANOVA ($p<0.05$). EAFHC 200 and the glibenclamide group rise more than the diabetic and EAFHC 100 groups.

EAFHC effect on lipid profile

EAFHC dose and the duration of administration has a significant effect to the percentage change total cholesterol of test animals ($p<0.05$). The rat given EAFHC for 5 d experience decline total cholesterol as seen in fig. 2. EAFHC dose and the duration of administration has no significant effect to the percentage change triglyceride of test animals ($p>0.05$) as seen in fig. 3.

EAFHC effect on ratio and histopathology pancreas

The pancreatic organ ratio was not significantly affected by EAFHC administration ($p>0.05$) as seen in fig. 4. Meanwhile, on the other side, EAFHC administration could repair the damage in the pancreatic caused by streptozotocin (fig. 5), as seen in histopatology score ($p<0.05$).

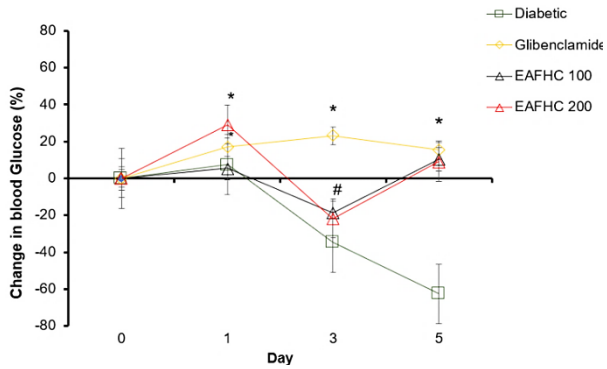

Fig. 1: Blood glucose changes levels±SE indiabetic animal models after administration of EAFHC. Each value is a mean of nine determination. *p<0.05 vs. Diabetic group; #p<0.05 vs. Glibenclamide group

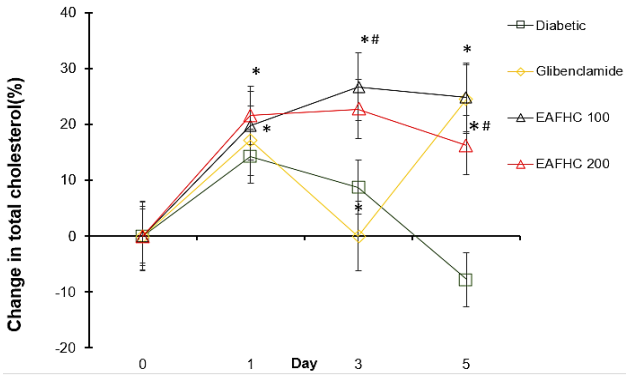

Fig. 2: Total cholesterol changes levels±SE in diabetic animal models after administration of EAFHC. Each value is a mean of nine determination. *p<0.05 vs. Diabetic group; #p<0.05 vs. Glibenclamide group

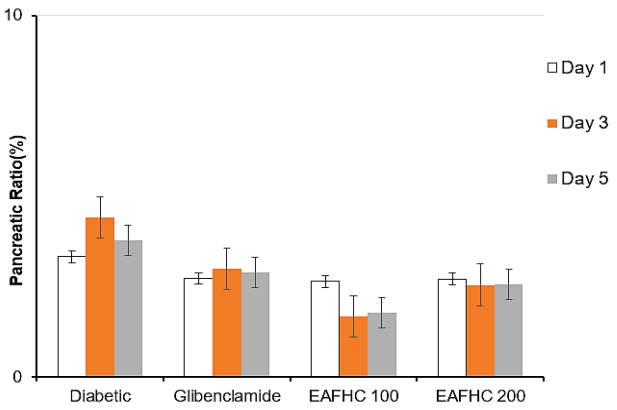

Fig. 3: Pancreatic ratio changes±SE in diabetic animal models after administration of EAFHC

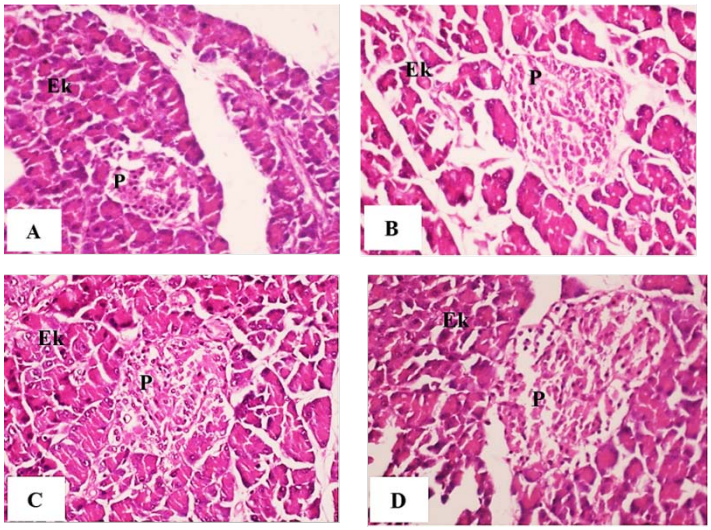

Fig. 4: Histology of pancreas WKY rat cells in streptozotocin-induced diabetes in day 5. Diabetic group (A), EAFHC 100 group (B), EAFHC 200 group (C), Glibenclamide group (D). H and E was used to stain 6-mm slices of pancreas at a magnification of 40x. Ek represented exocrine gland cells, P represented pancreatic islets,

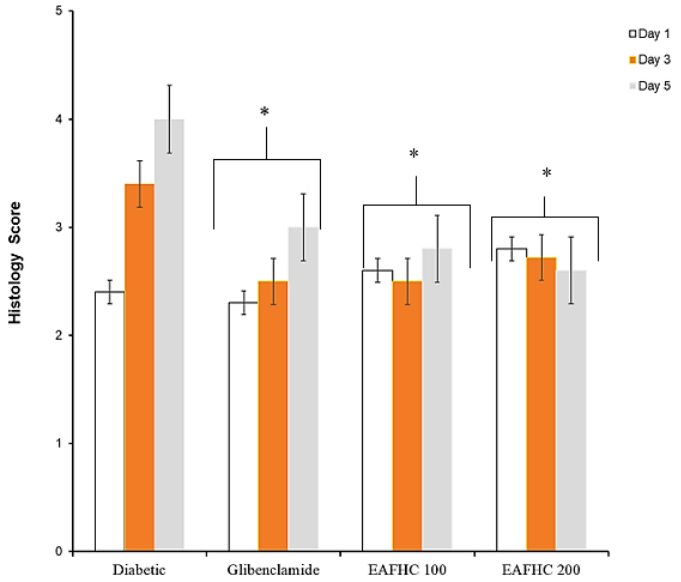

Fig. 5: Pancreatic histology score±SE in diabetic animal models after administration of EAFHC. Each value is a mean of nine determination. *p<0.05 vs. Diabetic group

DISCUSSION

Hibiscus sabdariffa L. originates from Africa and has been cultivated on a large scale in North Africa, India, Mexico, Indonesia, Thailand and Iran [22]. HSL has antioxidant activity by catching and neutralising radical free linked to the H atom in the OH group, making an electron donor of the hydrogen atoms and inactivating oxygen. H atoms will bind metal ions from radical free so that free radicals have balanced outer atoms and become neutral and non-reactive [23]. Antioxidants can hinder the production of free radicals and increase the production of potential nitrite oxide (NO), repair endothelial dysfunction and function of mitochondria in cells, and lower activity from NADPH oxidase enzyme [24]. In this study, antioxidants in EAFHC repair the damaged tissue by inhibiting the inflammatory process so the damaged pancreatic β cells can regenerate.

Streptozotocin was used as the control group. Streptozotocin binds to GLUT-2, facilitating STZ to enter cytoplasm pancreatic β cells, increasing depolarisation in mitochondria due to the influx of Ca2+ions followed by excess energy. This mechanism caused impaired insulin production, resulting in insulin deficiency and, consequently, blood glucose level increase [25].

Drugs used as group control in this research are glibenclamide. Glibenclamide is a sulfonylurea second generation that working mechanism by stimulating insulin secretion through closing potassium channel one sulfonylurea receptor (SUR1), so increasing the intracellular concentration of potassium ions and calcium ions will stimulate the release of granules containing insulin [24].

In this research, EAFHC can lower blood sugar in diabetes mellitus rats. Decreased blood sugar is due to the content of flavonoids and other compounds in EAFHC. According to literature, alkaloids, flavonoids, tannins, saponins, steroids and anthocyanins contained in EAFHC are capable of lowering blood glucose [13]. Anthocyanin compounds that exist in rosella can also hinder alpha-glucosidase enzymes present in the small intestine wall and alpha-amylase enzymes in the pancreas that is in the lumen of the intestine, resulting in disturbance in digestion and absorption glucose so that glucose

cannot absorbed by the intestine, consequently blood sugar decrease [24].

The decline in total cholesterol in animals after EAFHC administration in this study was caused by antioxidants contained in roselle. Decline total cholesterol caused by inhibition of LDL oxidation. Extracts of rosella calyx are capable of lowering total cholesterol through inhibition of LDL [14]. Flavonoids stabilise free radicals by reducing free radicals' activation energy and subsequently inhibiting LDL oxidation. Inhibition of LDL oxidation reactions causes cholesterol levels to decrease [26]. Another factor in reducing cholesterol levels by roselle extract is possible due to improving pancreatic β cells, resulting in high insulin production. Insulin inhibits hormone-sensitive lipase (HSL), stopping fat hydrolysis and converting fatty acids into phospholipids and cholesterol [27].

In this study, EAFHC does not significantly influence the reduction of triglycerides. An increase in triglycerides caused by high blood glucose results in excess blood sugar changed into lipids through lipogenesis, increasing triglycerides. In diabetes, insulin resistance occurs, which increases the activation of hormone-sensitive lipase (HSL) in adipose tissue. This increase causes excessive free fatty acids in the blood. Fatty acids are taken to the liver to be converted into triglycerides and become part of VLDL in the bloodstream. In a state of insulin resistance, VLDL contains quite a lot of triglycerides, so triglyceride levels in the blood tend to increase [28-30].

In this study, the pancreatic organ ratio was not significantly affected by variations in dose administration of the ethyl fraction acetate rosella calyx extract ($p>0.05$). Microscopic histopathology observation in the pancreas shows that streptozotocin-induced rats experienced damage to the pancreas, which is characterised by abnormal cells and degenerate endocrine gland cells in the islets of Langerhans accompanied by atrophy. The endocrine gland experiences partial degenerative damage and necrosis. This shows that EAFHC repairs the damage in pancreatic cells.

CONCLUSION

This study revealed that EAFHC could be an alternative medicine in managing blood sugar levels total cholesterol and improve pancreas function in associated model of diabetes mellitus hypercholesterolemia complications.

STATEMENTS OF ETHICS

All experiments complied with the European Council Directive on the Care and Use of Laboratory Animals (86/609/EEC). The protocol was approved by the Research and Ethics Committee of the Faculty of Pharmacy Universitas Andalas (No 33/UN.16.10. D. KEP-FF/2023).

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AUTHORS CONTRIBUTIONS

All authors provided significant contributions to the work addressed within this study. NS: Conceptualization, Methodology, Supervision, Funding acquisition; RA: Data curation, Writing-Original draft preparation, Writing-Review and Editing, Project administration; AA: Validation, Visualization, Investigation; CM: Formal analysis, Software, Validation.

CONFLICT OF INTERESTS

All authors declare that there is no conflict of interest regarding the publication of this article.

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