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Effect of Giving Ethanol Extract of Robusta Coffee Beans (Coffea canephora Pierre ex A. Froehner) Based on Immunohistochemical Examination of GLUT-4 Protein Expression in White Rats (Rattus norvegicus) Model of Diabetes Mellitus

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Abstract: Metabolic disorders are characterized by an increase in blood sugar due to a decrease in insulin secretion by pancreatic beta cells. Cells are unable to stimulate glucose absorption in muscle and fat tissue. The presence of GLUT-4 can increase the rate of glucose entry into insulin target tissues. The chlorogenic acid compound in robusta coffee beans is believed to lower blood glucose levels by activating GLUT-4. The aim of the research was to determine the effect of administering ethanol extract of robusta coffee beans *(Coffea canephora Pierre ex A. Froehner)* based on immunohistochemical examination of GLUT-4 protein expression in mice *(Rattus norvegicus)* with Type 2 Diabetes Mellitus. The research method included extraction by maceration with 96% ethanol: distilled water (3:7) solvent and immunohistochemical testing of the effect of ethanol extraction of Robusta coffee beans on mice. Rats were divided into 5 treatment groups, namely, Na-CMC 1%, metformin, 150 mg/kg BB, 300 mg/kg BB and 450 mg/kg BB and fed a high-fat diet for 6 months and immunohistochemical examination of skeletal muscle tissue was carried out. The results of immunohistochemical examination found that protein expression occurred in the positive control of coffee bean ethanol extract at doses of 150 mg/kg BB, 300 mg/kg BB and 450 mg/kg BB. The statistical test results show that the ethanol extract of robusta coffee beans at doses of 150 mg/kg BB, 300 mg/kg BB and 450 mg/kg BB is significant with the negative control Na-CMC 1% but with the positive control metformin the three doses of ethanol extract of robusta coffee beans are not significant. From the discussion above, it can be concluded that the ethanol extract of robusta coffee beans has an effect at doses of 300 mg/kg BB and 450 mg/kg BB

Keywords: GLUT-4; Immunohistochemistry; Insulin Resistance; Robusta Coffee

Introduction

World Health Organization (WHO) indicated that in 2000, around 150 million people worldwide suffered from Diabetes Mellitus caused by elevated blood glucose levels (hyperglycemia) due to insufficient insulin, and this number is projected to double by 2025. Population growth, aging, unhealthy diets, obesity, and

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lack of physical activity are major factors contributing to the increase in Type 2 Diabetes Mellitus patients. The most common form of diabetes mellitus is Type 2 DM, accounting for 90-94%, caused by insulin resistance. The highest prevalence rates are found in West Kalimantan province at 11.1%, while South Sulawesi province is approximately 4.6% (Rusman, 2022; WHO, 2020).

Insulin resistance is a disorder that affects the absorption of glucose in muscles and increases production by the liver, leading to hyperglycemia. Insulin resistance can affect the function of other organs, such as blood vessels (causing vasoconstriction/hypertension), the brain (increasing calories), the pancreas (reducing beta cell mass), and bones (reducing bone mass and strength (Sari et al., 2021; Wang et al., 2020). Normally, after eating, the secretion of insulin hormone and glucose absorption increase, so that blood glucose levels can be maintained within normal limits. However, in cases of obesity, the body becomes less responsive to insulin. Elevated blood glucose levels in Type 2 DM patients are more often caused by insulin resistance in peripheral tissues, leading to the failure of most glucose to enter the cells (Hasanuddin et al., 2021).

The physiological process of glucose uptake into cells is regulated by insulin and the rate of glucose transport through a specific protein associated with the plasma membrane, facilitated by glucose transporters (GLUTs). According to Thorens & Mueckler (2010), the well-known glucose transporter is GLUT-4. GLUT-4 is a glucose transporter for glucose distributed in skeletal muscle tissue and adipose tissue (Aluko et al., 2020; Hasanuddin et al., 2021; Hayat et al., 2024; Lai & Hiu, 2015). Glucose transporter 4 (GLUT-4) is a specific protein that facilitates glucose transport responsive to insulin in muscle and adipose tissues. GLUT-4 translocates to the plasma membrane in response to insulin. GLUT-4 is known as the primary glucose transporter and can regulate glucose stimulated by insulin secreted by beta cells as glucose sensors (Ghaedi et al., 2020; Hasanuddin et al., 2021, 2022; McCarty & Assanga, 2018)

Immunohistochemistry is a process used to detect antigens (proteins, carbohydrates, etc.) in cells of tissues through the microscopic binding reaction of antibodies to antigens in tissues. Immunohistochemistry is often used to identify cell proliferation processes and cell apoptosis. It is also commonly utilized in basic research to determine the distribution and location of biomarkers or expressed proteins in various tissues of the body (Feitosa et al., 2018).

One of the dominant polyphenolic compounds found in coffee is chlorogenic acid. Chlorogenic acid in coffee plays a role in lipid and glucose metabolism, thus helping to prevent liver steatosis, cardiovascular diseases, diabetes, and obesity (Naveed et al., 2018). The active component of coffee, chlorogenic acid, can effectively aid in regulating blood glucose levels, inhibit intestinal glucose absorption, and improve insulin sensitivity.

The purpose of this study is to determine the effect of administering ethanol extract of robusta coffee beans (Coffea canephora Pierre ex A. Froehner) based on the examination of GLUT-4 Protein Expression through Immunohistochemistry in type 2 Diabetes Mellitus rats (Rattus norvegicus)

Method

Sampling

The study utilized robusta coffee beans (Coffea canephora Pierre ex A. Froehner) sourced from Gandangbatu Village, Gandangbatu Silanan District, Tana Toraja Regency, with coordinates at South Latitude (S) 3O14'40.632" and East Longitude (E) 1190 49' 08.7312". The selected coffee beans were red ripe and handpicked individually, with manual separation of the skin and seeds.

The equipment utilized included a maceration vessel, sample cup, Erlenmeyer flask, chemical glassware (Pyrex), measuring cup (Pyrex), glucometer (Elva Sense), hot plate, rat cage, slide basket, 500 µL micropipette, tissue processing machine (Automatic Tissue Processor), digital microscope, capillary tube, sample rack, Thermo Scientific Indiko digital scale, blue tips, oral sonde spoit, and water bath.

The materials employed comprised aluminum foil, robusta coffee beans (Coffea canephora Pierre ex A. Froehner), deck glass, 96% ethanol (C_2H_5OH), 70% ethanol (C_2H_5OH), 80% ethanol (C_2H_5OH), Meyer's hematoxylin, N-hexane, sodium carboxymethyl cellulose (Na-CMC) 1%, object glass, high-fat diet feed, liquid paraffin (Paraffinum Liquidum), serum samples, Elva Sense strips, and metformin tablets ($C_4H_{11}N_5$).

Assay for the activity of GLUT-4 protein expression.
Administering a high-fat diet to experimental animals

Twenty male Wistar strain white rats, aged 3 months and weighing between 150-200 g, were utilized for this study. The rats were acclimatized under controlled room temperature conditions and subjected to a light-dark cycle. Initial blood glucose levels were measured prior to the commencement of the study. Induction was carried out by administering a high-fat diet for 24 weeks. The induction phase was marked by the onset of obesity, defined as weight gain exceeding 200 g and an increase in blood glucose levels above 100

mg/dL. Following induction, the rats were subjected to a 2-week trial of ethanol extract of robusta coffee beans (Coffea canephora Pierre ex A. Froehner). High-fat diet feeding was continued throughout the trial period, and subsequently, immunohistochemical examination was conducted.

Immunohistochemical analysis of the samples

The skeletal muscle tissue used was the soleus muscle. For tissue collection, the rats were first anesthetized using inhalation ether, followed by cervical dislocation. The collected muscle tissue was then placed in 10% formalin buffer for fixation. After fixation, tissue processing was performed to produce paraffin blocks. Subsequently, the following steps were carried out for the immunohistochemistry (IHC):

Deparaffinization process after slide preparation

H_2O_2 blocking: Slides were immersed in 0.3% H_2O_2 solution in methanol for 15 minutes, followed by rinsing with distilled water: Washed 3 times for 5 minutes each with Phosphate Buffer Saline (PBS) pH 7.4; Background sniper treatment for 10 minutes; Primary antibody incubation: GLUT-4 antibody at dilutions of 1:250 and 1:500 (in PBS) for 24 hours at room temperature; Washed 3 times for 5 minutes each with PBS pH 7.4; Secondary antibody (biotinylated) incubation using Trekkie universal link, at room temperature for 20 minutes; Washed 3 times for 5 minutes each with PBS pH 7.4; Betazoid DAB chromogen incubation for 15 minutes at room temperature; Washed with distilled water; Mayer's hematoxylin staining at a ratio of 1:3 in distilled water for 30 seconds; Rinsed with running water for 30 seconds; Dehydration for 15 minutes (successively using 30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 95% ethanol, absolute ethanol, and xylene solution); and Mounting process.

Photomicroscopy analysis

For each sample, 3 random sections of tissue were selected from a total of 9 tissue sections on each slide. Subsequently, 6 photomicrographs were taken from each selected section at a magnification of 400x.To quantify the expression of the GLUT-4 protein:

Each photograph was quantified using the IHC profile plugin in ImageJ software. This software utilizes color deconvolution principles and pixel-by-pixel image analysis to obtain scores automatically (Varghese et al., 2014).

The results from Image software were scored as follows: negative (0), weak positive (1), positive (2), and strong positive (3). The data were then presented as the mean values.

Result and Discussion

This research utilized robusta coffee bean samples (Coffea canephora Pierre Ex. A Froehner) extracted through maceration using 96% ethanol:aquadest (3:7) solvent, yielding extract yield (Table 1).

The study began by inducing rats using a high-fat diet (HFD) for 6 months. The HFD used had a composition of 39.57% fat, 14.52% protein, 2.82% crude fiber, 3.39% ash, and 6.85% water. Inducing rats with HFD can lead to insulin resistance, where the diet used contains 30-50% fat of total calories. One of the parameters used was the measurement of blood glucose and immunohistochemical examination.

Table 1. Result Data of Robusta Coffee Beans Yield (Coffea canephora Pierre Ex. A Froehner)

Sample Weight (g)	Extract Weight (g)	Yield (%)
500	48.78	9.756

Table 2. Data of Mean Measurement Results of Fasting Blood Glucose (FBG) Before, After Induction, and After Extract Administration

Parameter	Initially	After the diet induction HFD	After the administration of the extract
Mean value ± SD	86.20 ± 10.401	123.27± 9.881	99.60 ± 10.716
Mean P	<0.05		

Table 3. Data of Mean IHC Profile Cytoplasm Stained (FBG) Before, After Induction, and After Extract Administration

Parameters	Initially	After the administration of the extract	STD
Mean value ± SD	86.20 ± 10.401	99.60 ± 10.716	3.00 ± 1.435
Mean P	<0.05		

Based on the study conducted, the blood glucose level measurements obtained were as follows: the initial blood glucose level ranged from 86.20 ± 10.401, the blood glucose level after induction with a high-fat diet was 123.27 ± 9.881, and the blood glucose level after extract administration was 99.60 ± 10.716.

The average blood glucose levels obtained from Table 3 show that after the initial treatment, for Group I

given Na-CMC 1%, the initial blood glucose level was 102 mg/dL. After induction with a high-fat diet, the glucose level increased to 112.3 mg/dL, and after being given the negative control Na-CMC 1%, the glucose level remained elevated at 120 mg/dL. This indicates that when induced with a high-fat diet, the blood glucose levels increased. This is consistent with the research by Heydemann (2016), where administering a high-fat diet for 24 weeks can lead to insulin resistance.

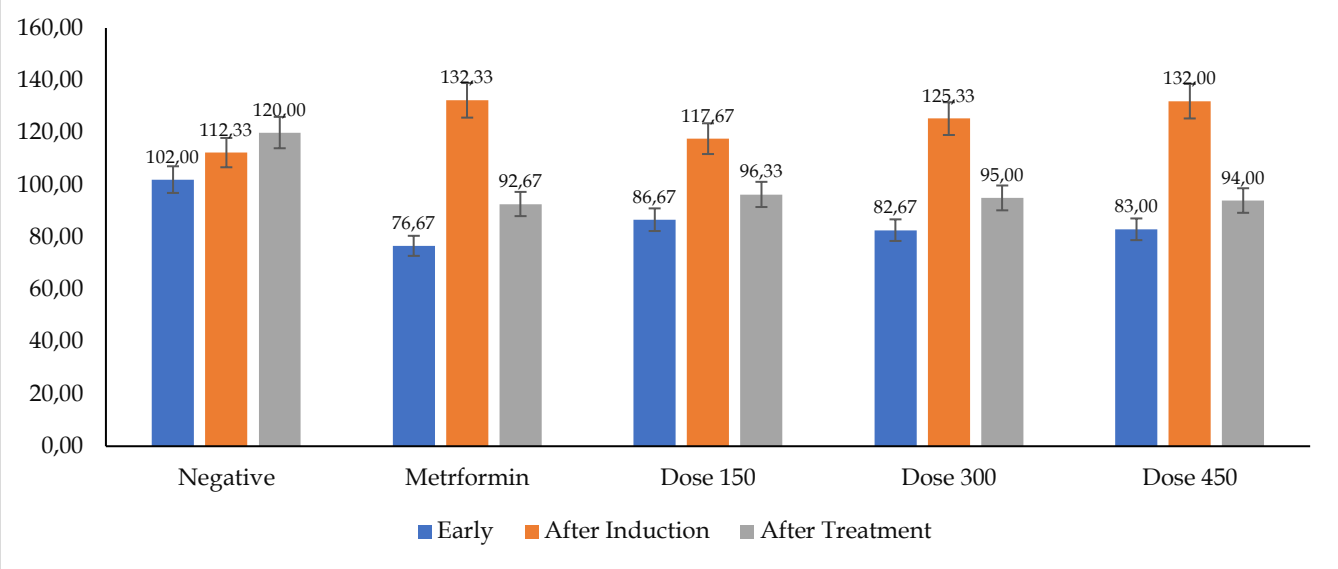

Figure 1. Graph of Mean Fasting Blood Glucose (FBG) Levels Before, After Induction, and After Extract Administration Normal blood glucose levels for rats are reported to be between 70 - 110 mg/dL (Ganong, 1995).

The average blood glucose data for Group II, administered with metformin, showed an initial blood glucose level of 76.67 mg/dL. After induction with a high-fat diet, the blood glucose level increased to 132.33 mg/dL, and after being given metformin, the blood glucose level decreased to 92.67 mg/dL. This indicates that after the administration of metformin, the blood glucose level decreased. This is because metformin is one of the oral antidiabetic drugs of the biguanide class, which works by increasing the body's sensitivity to insulin produced by the pancreas and reducing hepatic glucose production through AMP-activated protein kinase enzyme activity, as well as increasing glucose uptake stimulants by skeletal muscles (Katzung et al., 2017; Trevor et al., 2010).

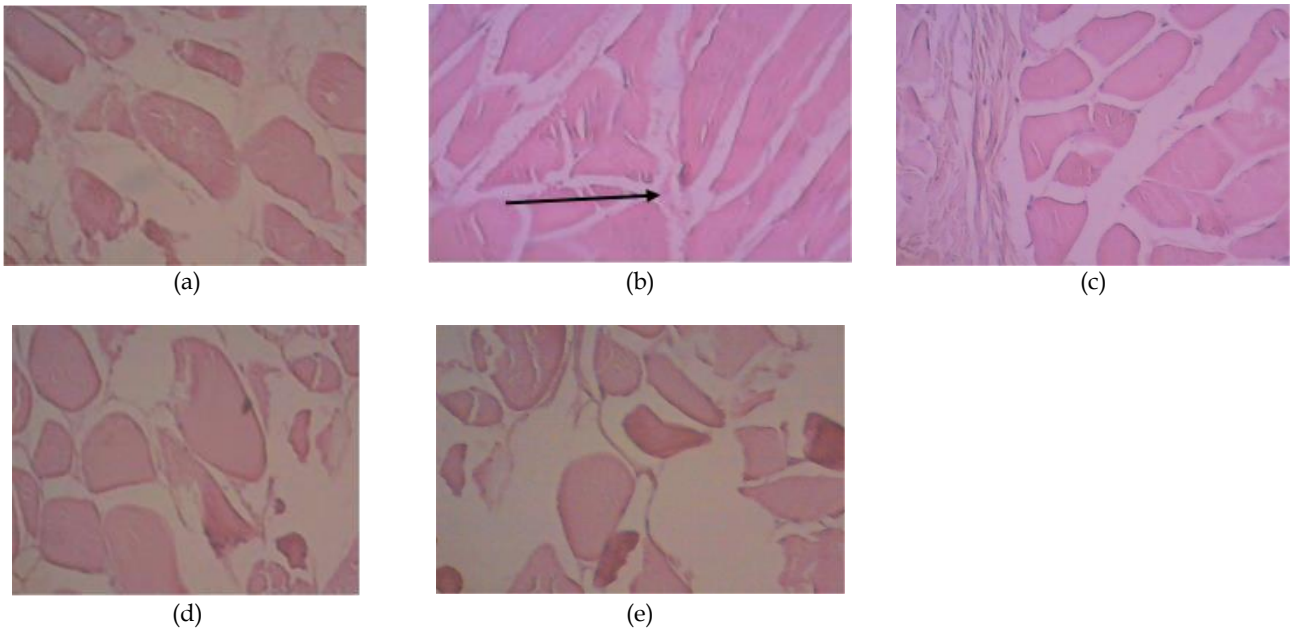

Figure 2. Characteristic morphology: (a) Control (-) Na-CMC 1%; (b) control (+) Metformin; (c) Extract 150 mg/kg BW; (d) Extract 300 mg/kg BW; and (e) Extract 450 mg/kg BW.

The average blood glucose data for Group III, given ethanol extract of robusta coffee beans at a dose of 150 mg/kg BW for 14 days, showed a blood glucose level of 96.3 mg/dL. Group IV, given ethanol extract of robusta coffee beans at a dose of 300 mg/kg BW, showed a blood glucose level of 95 mg/dL. Group V, given ethanol extract of robusta coffee beans at a dose of 450 mg/kg BW, showed a blood glucose level of 94 mg/dL. This indicates that the higher the extract concentration, the lower the average blood glucose level. This is influenced by chlorogenic acid, which has a mechanism in reducing intracellular hyperglycemia and acts as a polyphenolic compound that functions as an antioxidant in coffee. The antioxidant content of coffee has also been reported by several researchers to be chlorogenic acid, which is an ester of caffeic acid and quinic acid, responsible for its antioxidant properties. Chlorogenic acid can increase insulin sensitivity, especially in muscle tissues, through the quinide compounds within it (Damayanti et al., 2023; Kurnia, 2021).

Based on the results of immunohistochemical examination, it was found that protein expression occurred in positive controls of robusta coffee bean ethanol extract at doses of 150 mg/kg BW, 300 mg/kg BW, and 450 mg/kg BW. Statistical tests revealed that robusta coffee bean ethanol extract at doses of 150 mg/kg BW, 300 mg/kg BW, and 450 mg/kg BW were significant compared to the negative control Na-CMC 1%, but not significant compared to the positive control metformin. This indicates that the higher the extract given, the more positive tissue expression occurs, both in muscle and fat tissues. GLUT-4 is highly present in tissues that absorb the most glucose from the blood, namely skeletal muscle and adipose tissue. This is consistent with the administration of coffee bean extract at doses of 300 mg/kg BW and 450 mg/kg BW, which affects GLUT-4, which is sensitive to glucose uptake regulated by insulin. Insulin works to maintain glucose homeostasis by suppressing gluconeogenesis and glycogenolysis processes (Barthel & Schmoll, 2003; Hatting et al., 2018).

Additionally, insulin increases glucose uptake by peripheral tissues, especially adipose tissue and skeletal muscle, with the help of glucose transporter (GLUT) (Chadt & Al-Hasani, 2020; Ferrannini et al., 2018; Klip & Pâquet, 1990). In this case, facilitated transport occurs where glucose can passively diffuse into adipose tissue without requiring ATP, using GLUT-4. GLUT-4 reacts quickly to increased plasma insulin levels by increasing glucose transport by 20-30 times. Without glucose stimulation, 90% of GLUT-4 is sequestered within skeletal muscle tissue cellsAdipose tissue is characterized by vesicles with double-layered lipid membranes. On the cell surface, GLUT-4 facilitates the passive diffusion of circulating glucose when the glucose concentration in skeletal muscle and adipose tissue decreases (Feitosa et al., 2018).

Conclusion

Based on the results of statistical data analysis and discussion, it can be concluded that the ethanol extract of robusta coffee beans (Coffea canephora Pierre ex A. Froehner) has an effect on the expression of GLUT-4 protein at doses of 300 mg/kg BW and 450 mg/kg BW, which are significantly different from the negative control Na-CMC 1%. However, with the positive control metformin, the three doses of robusta coffee bean ethanol extract did not show significant differences.

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Conflicts of Interest
The authors declare no conflict of interest.

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