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RESEARCH ARTICLE

Potency of *Clitoria ternatea* L. Ethanol Extract Against IFNγ and GLUT4 Protein in Type 2 Diabetes Mellitus Rat Model

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Abstract

Diabetes Mellitus (DM) is a chronic disease caused by genetics, lifestyle, and immunology. Type 2 DM is caused by resistance to insulin, which causes blood sugar to not be stored in glycogen. *Clitoria ternatea* L. (Fabaceae) flower contains a lot of anthocyanins and has long been used in various traditional medicines. They are believed to treat fever, inflammation, and diabetes. Anthocyanins have antidiabetic, anticancer, anti-inflammatory, and anticancer properties. This study aimed to evaluate anti-DM of *Clitoria ternatea* L. flower extract (CTE) in DM model rats with parameters such as liver weight ratio, IFNγ, and GLUT4 protein expression in pancreas and femoral muscle. Rats with a streptozotocin-induced (STZ) DM model were used to test the effects of oral administration of CTE at doses of 200, 400, and 800 mg/kg BW, glybenclamide (0.45 mg/kg BW), and simvastatin (0.9 mg/kg BW). After blood sugar levels were >200 mg/dL, the rats were given various doses of CTE and tested for protein expression of IFNγ and GLUT4 in femoral muscle by immunohistochemistry (IHC) method. Data analysis used ANOVA and continued with Tukey's post hoc test. Results showed that CTE could increase liver ratio while decreasing IFNγ activation at CTE 400 mg/kg BW and upregulating GLUT4 at 400 mg/kg BW. CTE has an anti-DM activity potential for diabetes mellitus treatment due to its anthocyanin content.

Keywords: *Clitoria ternatea*, Diabetes Mellitus, IFNγ, Immunohistochemistry, GLUT4

1. Introduction

Diabetes Mellitus (DM) is a chronic condition in which there is an increase in blood glucose levels. It can cause the body to not produce insulin, not produce adequate insulin, or not use insulin effectively. The estimated global prevalence of DM in 2019 is 9.3% (463 million people) and will increase to 10.2% (578 million people) in 2030. There are two main types of DM: type 1 and type 2. Type 1 DM is a disease due to the immune system, which leads the immune system to the destruction of the insulin-producing pancreatic beta cells that causes insulin deficiency in human. Type 2 DM is a disease due to insulin resistance, and a lack of appropriate compensation by the beta cells causes insulin deficiency. Type 2 DM mostly occurs because of lifestyle, although some cases are genetically inherited[1]. Treatment of type 2 DM involves lifestyle changes and taking medications to reduce blood glucose. Most DM drugs are synthetic compounds that have side effects on people who take them, such as metformin, insulin secretion sulfonylureas and meglitinides, Glucagon Like Peptide-1 Receptor Agonist (GLP-1RA), etc[2]. The drugs to treat type 2 DM can be associated with certain drawbacks, so there is a need to find alternatives to reduce blood glucose in patients with type 2 DM.

Glucose Transporter (GLUT) is a protein that plays a role in the transport of glucose into cells. There are 2

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types of GLUT, namely sodium-dependent GLUT and facilitative GLUT. GLUT1 is included in the facilitative GLUT, which is found ubiquitously in all tissues in the body and helps in the entry of glucose into cells[3]. GLUT1 is a hydrophobic transmembrane protein that is 50% found in the lipid bilayer[4]. GLUT4 is a transmembrane protein that plays an important role in glucose homeostasis in cells. GLUT4 is expressed in skeletal muscle and adipose tissue[5]. GLUT4 regulation is influenced by insulin which has an impact on the distribution of GLUT4 on the cell surface which will affect glucose entering cells[6]. Interferon γ (IFNγ) is an inflammatory cytokine that has a role in the fibrosis of inflamed tissues. IFNγ is the most potent activator of macrophages, which can identify and phagocytose microbes and cancer cells[7].

Clitoria ternatea L. is a plant species from the Fabaceae family. Its flowers have a bright blue color, and due to their colors, they are used for various decorative items and as natural coloring agents around the world. *C. ternatea* contains flavonoid glycosides such as rutin, delphidin, kaempferol, quercetin, and malvidin[8]. This plant has been documented in several properties such as antioxidant, anti-inflammation, antidiabetic, hypolipidemic, anticancer, and anti-platelet-aggregation[9]. *C. ternatea* is an Indonesian endemic plant from Ternate because the specimen identified by Linnaeus came from Ternate[10]. In this study, we aim to evaluate the administration of *C. ternatea* flower extract towards the ratio of body weight and pancreas weight, and on the expression of IFNγ and GLUT4 protein.

2. Materials and Methods

2.1 Extract Preparation

C. ternatea L. was discovered in Kampung Herbal, Sukolilo Village, Prigen District, Pasuruan, East Java, Indonesia. The Indonesian Institute of Sciences (LIPI) in Bogor was in charge of plant identification. The extraction was carried out by Industri Obat Tradisional (IOT) PT FAST, and it was standardized utilizing GMPs (GMP). The powdered telang flower extract was produced with a Certificate of Analysis using a 70% ethanol as the solvent. Extract was obtained by maceration and used additional material namely lactose (No. Batch: 001103211072)[11].

2.2 Animals Experimental

The experimental animals used were male Sprague Dawley rats, aged ± 6 weeks, weighing between 120 and 140 g. Rats were maintained in a temperature room (± 25 °C) on a 12 h light-dark natural cycle. Rats were fed with a standard diet and water ad libitum for 7 days[11]. Rats were fed with High Fat Diet (HFD) of 5.5% crude fiber, 18% crude protein, and 50% crude fat (PT Indoofeed) while the standard diet had 7.37% crude fat and 0.01% Propylthiouracil (PTU, Dexa Medica) in drunk water[11,12]. Rats were given HFD and PTU for 28 days to induce dyslipidemia in them[11-13].

Streptozotocin (STZ) was administered intraperitoneally once (Sigma Aldrich SO130) and dissolved in 0.1 M citrate buffer (pH 4.5) at 60 mg/kg of body weight. Nicotinamide (NA, Sigma Aldrich-N0363) dissolved in normal saline at a dose of 120 mg/kg of body weight was administered intraperitoneally (ip) for 60 minutes. After five days, utilizing Autocheck blood glucose, 12 h Fasting Blood Glucose (FBG) 250 mg/dL demonstrated rats suffered DM[14]. The rats were treated with CTE (200, 400, 800 mg/kg of BW), glibenclamide at 0.45 mg/kg of BW (Generic, GKL9520905004A2), and simvastatin at 0.9 mg/kg of BW (Generic, GKL131670271A), while distilled water was given as a negative control for 28 days after the rats' dyslipidemia and diabetes were confirmed[11,15].

There were eight Groups of four rats each in the DM rat models. Rats in Group I - the negative control (NC) were left untreated. HFD and PTU were administered to Group II's DM rats acting as a positive control (PC). CTE was administered to rats in Group III, IV, and V at dosages of 200, 400 and 800 mg/kg BW, respectively. Glibenclamide was administered to Group VI DM rats at a dose of 0.45 mg/kg BW, while simvastatin was administered to Group VII DM rats at a dose of 0.9 mg/kg BW. Rats in Group VIII DM received glibenclamide and simvastatin in combination. These studies were conducted with the approval of the Maranatha Christian University (147/KEP/VI/2021).

2.3 Immunohistochemistry Assay

Observations with immunohistochemistry were carried out descriptively by looking at the population and the appearance of the levels of antigen (Ag) and antibody (Ab) beta cells that underwent changes. The principle of the IHC test is a positive reaction to the

presence of insulin in beta cells indicated by a brown color change in these cells. Pancreatic tissue was cut along ± 5 mm and soaked in 4% paraformaldehyde fixative, then paraffin blocks were made. Paraffin blocks were sliced at 4 µm thickness for IHC staining. IHC staining for femoral muscle using IFNγ, GLUT4 polyclonal primary antibody (Elabsci E-AB-65910, Elabsci E-AB-30268) at 370 C for one-night. Protein target visualization utilized Two-step plus Poly-HRP Anti Mouse/Rabbit IgG Detection System (with DAB solution) (Elabsci, E-IR-R217). Images were analyzed qualitatively using a light microscope[11,16,17].

2.4 Statistical Analysis

All the data are presented as the mean ± standard deviation. The data were analyzed using One Way ANOVA test followed by the Tukey Post Hoc test. P-values < 0.05 were considered significant.

3. Results

3.1 Effect of CTE on DM Rat Model Body Weight

On day 28 following the administration of various doses of CTE, body weight levels of DM and dyslipidemia rats were measured (Figure 1). CTE 800 mg/kg BW was the optimal CTE dose for reducing BW.

3.2 Effect of CTE on Liver Ratio

Figure 2 shows the effect of CTE on the liver weight ratio of the DM rat Group. Group I (negative control) had a liver ratio score of 4.55. In the Group II (positive control), the liver ratio was 2.68. Group III with treatment of 200 mg/kg BW of CTE had the lowest liver weight ratio among the three CTE treatments, which was 2.93. Group IV with treatment of CTE as much as 400 mg/kg BW had a liver ratio of 3.89. The highest liver weight ratio among the three CTE treatments was Group V (800 mg/kg BW CTE) with a score of 3.93. When compared with drug administration, Group V was still higher than Group VIII (Glibenclamide 0.45 mg/kg BW and Simvastatin 0.9 mg/kg BW) which had a liver weight ratio score of 3.60.

3.3 IFNγ Protein Expression in Pancreas

Figure 3 demonstrates that the positive control (44.848%) had a field of view after receiving STZ, HFD. The visual field of CTE was lowest at a dose of 400 mg/kg BW (24.744%), whereas doses of 200 mg/

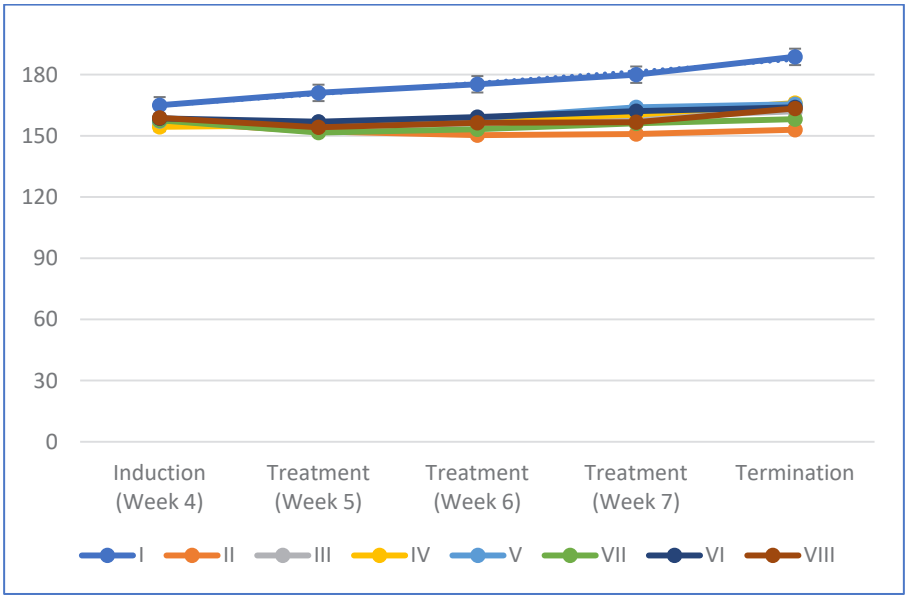

Figure 1. The effect of CTE on DM rat model body weight.

*The mean and standard deviation of the data are displayed. I: negative control; II: positive control; III: positive control + CTE 200 mg/kg BW (CTE1); IV: positive control + CTE 400 mg/kg BW (CTE2); V: positive control + CTE 800 mg/kg BW (CTE3); VI: positive control + Glibenclamide 0.45 mg/kg BW; VII: positive control + Simvastatin 0.9 mg/kg BW; VIII: positive control + Glibenclamide 0.45 mg/kg BW and Simvastatin 0.9 mg/kg BW.

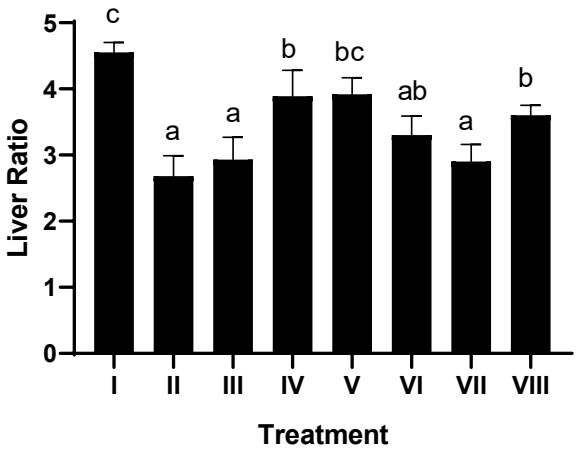

Figure 2. The effect of CTE on liver ratio of DM rat model.

*The mean and standard deviation of the data are displayed. Based on Tukey's HSD Post Hoc Test ($p<0.05$), different letters (a, ab, b, bc, and c) denote significant differences among various Group treatments. I: negative control; II: positive control; III: positive control + CTE 200 mg/kg BW (CTE1); IV: positive control + CTE 400 mg/kg BW (CTE2); V: positive control + CTE 800 mg/kg BW (CTE3); VI: positive control + Glibenclamide 0.45 mg/kg BW; VII: positive control + Simvastatin 0.9 mg/kg BW; VIII: positive control + Glibenclamide 0.45 mg/kg BW and Simvastatin 0.9 mg/kg BW.

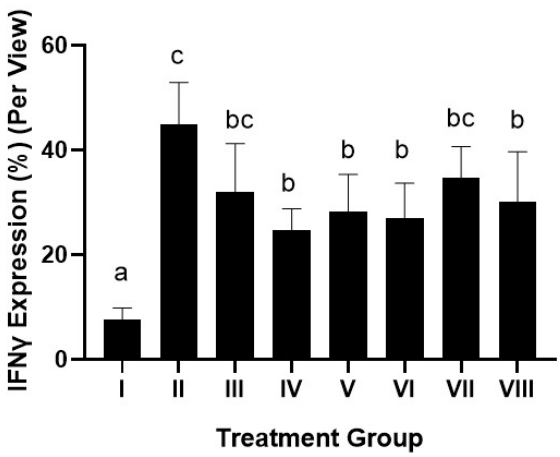

Figure 3. The effect of CTE on pancreatic IFNγ expression of DM rat model.

*The mean and standard deviation of the data are displayed. Based on Tukey's HSD Post Hoc Test ($p<0.05$), different letters (a, b, bc, and c) denote significant differences among various Group treatments. I: negative control; II: positive control; III: positive control + CTE 200 mg/kg BW (CTE1); IV: positive control + CTE 400 mg/kg BW (CTE2); V: positive control + CTE 800 mg/kg BW (CTE3); VI: positive control + Glibenclamide 0.45 mg/kg BW; VII: positive control + Simvastatin 0.9 mg/kg BW; VIII: positive control + Glibenclamide 0.45 mg/kg BW and Simvastatin 0.9 mg/kg BW.

kg BW exhibited visual fields of 32.009%. This result showed that the CTE treatment could lower pancreatic IFN*γ* expression.

The presence of IFNγ protein is indicated by brown color (Figure 4). In the PC, dark brown control cells were seen (Figure 4.II). The darker color can be interpreted as the higher expression of IFNγ protein (Figure 4.II). The treatment at concentrations of 400 and 800 mg/kg BW showed a lower intensity of the brown hue, indicating that the CTE could decrease the regulation of IFNγ.

3.4 GLUT4 Protein Expression in Femoral Muscle

Brown color is a sign that GLUT4 protein is detected (Figure 5). The PC were less brown and withered (Figure 5.II). A deeper hue could indicate that there is more GLUT4 protein present (Figure 5.IV). Treatment at 400 mg/kg BW resulted in a more intense brown color, demonstrating that CTE has the ability to upregulate GLUT4.

According to Figure 6, CTE treatment increased GLUT4 expression of femoral muscle (80.08%). The most active to increase GLUT4 expression was CTE 400 mg/kg BW, and it was comparable to glibenclamide 0.45 mg/kg BW.

4. Discussion

In this study, the test rats in Group II-VIII were induced by streptozotocin (STZ). The PC Group showed the lowest liver ratio among other Groups, which was 2.68 of liver ratio (Figure 1). This finding is in line with previous study that stated STZ could undermine the ability of the pancreas to produce insulin and encourages damage to its cells[18]. Osmotic diuresis, which should happen because of elevated blood glucose levels and result in a loss of glucose and an abundance of urine, should also increase water consumption.

IFNγ, a Th1 proinflammatory cytokine, is crucial for organ-specific autoimmune disorders as well as defense against intracellular and viral infections. IFNγ has the

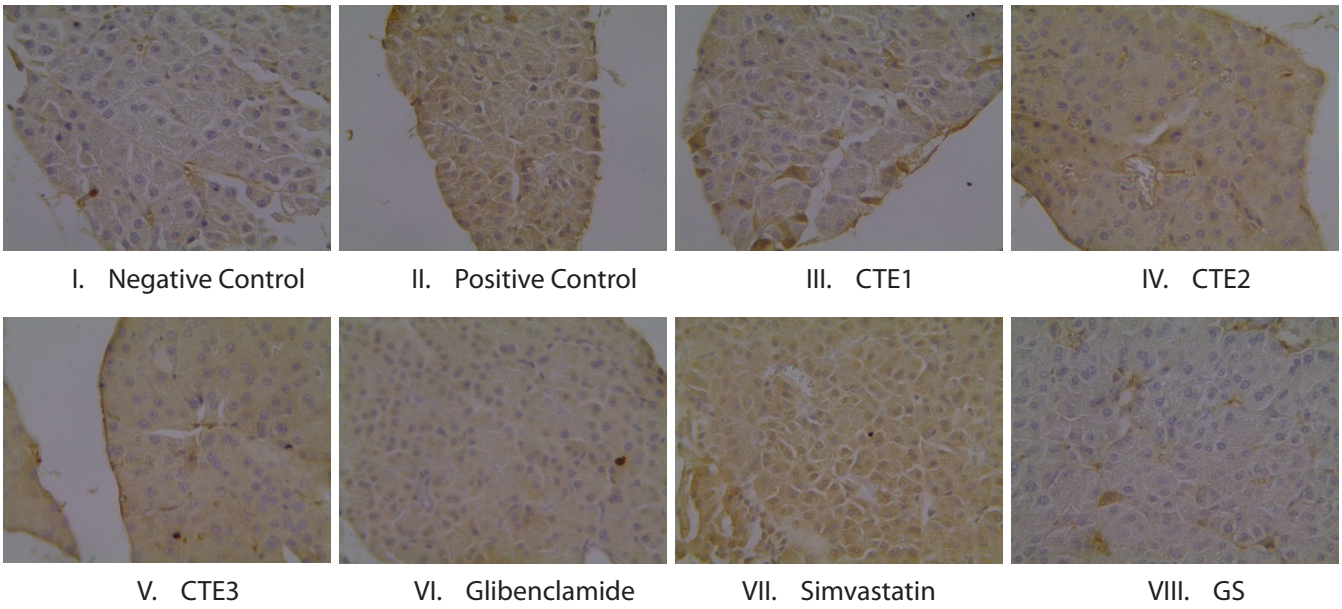

Figure 4. The effect of CTE on pancreatic IFNγ expression by IHC staining.

*I: negative control; II: positive control; III: positive control + CTE 200 mg/kg BW (CTE1); IV: positive control + CTE 400 mg/kg BW (CTE2); V: positive control + CTE 800 mg/kg BW (CTE3); VI: positive control + Glibenclamide 0.45 mg/kg BW; VII: positive control + Simvastatin 0.9 mg/ kg BW; VIII: Glibenclamide 0.45 mg/kg BW and Simvastatin 0.9 mg/kg BW.

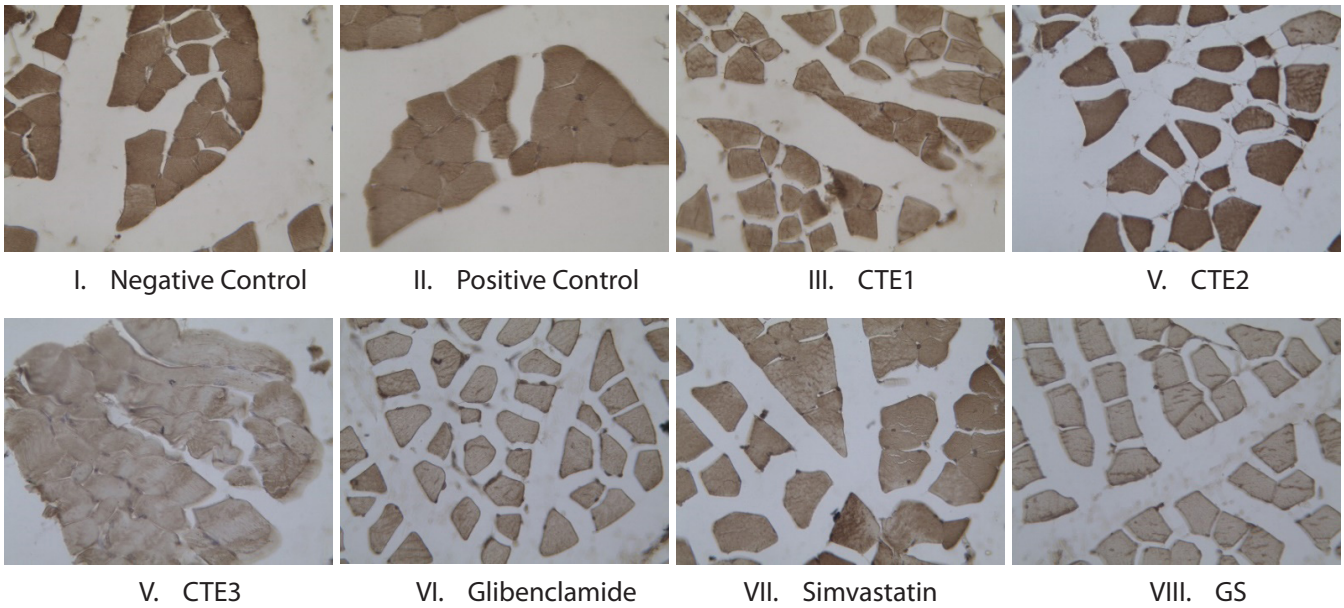

Figure 5. The effect of CTE on femoral muscle GLUT4 expression by IHC staining.

* I: negative control; II: positive control; III: positive control + CTE 200 mg/kg BW (CTE1); IV: positive control + CTE 400 mg/kg BW (CTE2); V: positive control + CTE 800 mg/kg BW (CTE3); VI: positive control + Glibenclamide 0.45 mg/kg BW; VII: positive control + Simvastatin 0.9 mg/kg BW; VIII: positive control + Glibenclamide 0.45 mg/kg BW and Simvastatin 0.9 mg/kg BW.

ability to activate macrophages and dendritic cells, upregulate the production of Major Histocompatibility Complex (MHC) molecules, and enhance the function of antigen-presenting cells. Adults who have latent autoimmune diabetes and T2DM have been linked to high levels of IFNγ[19].

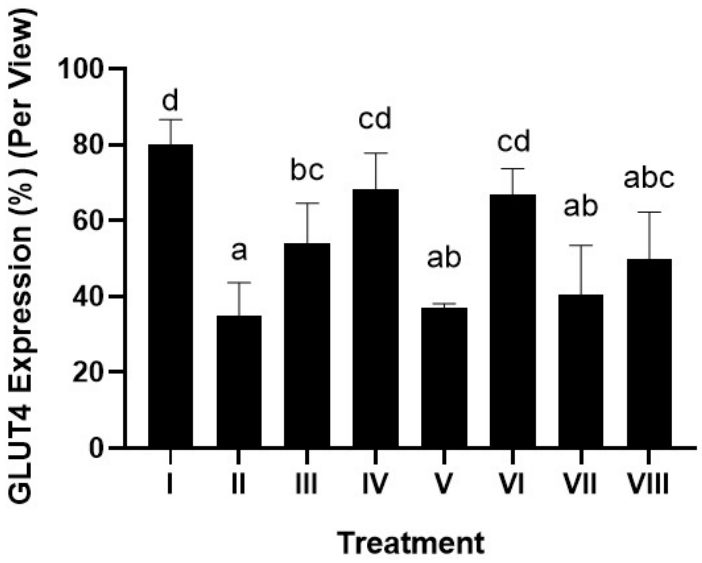

Figure 6. The effect of CTE on femoral muscle GLUT4 of DM rat model.

*The mean and standard deviation of the data are displayed. Based on Tukey's HSD Post Hoc Test ($p<0.05$), different letters (a, ab, abc, bc, cd, and d) denote significant differences among various Group treatments. I: negative control; II: positive control; III: positive control + CTE 200 mg/kg BW (CTE1); IV: positive control + CTE 400 mg/kg BW (CTE2); V: positive control + CTE 800 mg/kg BW (CTE3); VI: positive control + Glibenclamide 0.45 mg/kg BW; VII: positive control + Simvastatin 0.9 mg/kg BW; VIII: positive control + Glibenclamide 0.45 mg/kg BW and Simvastatin 0.9 mg/kg BW.

This study evaluated the IFNγ protein expression and discovered that the positive control Group of rat had highest IFNγ levels, which indicated significant inflammation. In contrast, rat treated with CTE had significantly lower IFNγ expression (Figures 3 and 4). These findings implied that administering CTE to diabetic rats reduced inflammation linked to type 2 DM. A previous study mentioned that significantly lower levels of IFNγ may possibly be responsible for the low frequency of apoptotic islet cells in treated animals, given that IFNγ is essential for type 2 DM-related inflammation and associated b-cell apoptosis. A different study also reported that the level of IFNγ in diabetic patients were higher than in healthy subjects. They also claimed that IFNγ destroyed beta cells in the islets of Langerhans, which led to insulin resistance, which is a factor in the development of diabetes[20]. These data enlarge findings regarding the potential of CTE to reduce IFNγ levels.

In the AMP-activated protein kinase (AMPK) pathway, GLUT4 is an insulin-regulated glucose transporter[21]. Increased insulin sensitivity and glucose tolerance may result from high levels of GLUT4 expression in adipose tissue. Disruption of GLUT4 expression in mice results in insulin resistance and over exoression of GLUT4 ameliorates diabetes in the db/db mouse model. For the maintenance of glucose homeostasis, GLUT4 in muscle and adipose tissue is essential. Greater insulin resistance is caused by the specific deletion of GLUT4 from muscle or adipocytes than by the isolation of the insulin-signaling proteins from these tissues. In the study conducted, the PC showed the lowest GLUT4 expression among others. This condition is in line with the outcomes of prior studies, where GLUT4 expression is low in subjects suffering from type 2 DM, especially with poor insulin resistance conditions[22]. The analysis results showed that the CTE treatments (200, 400, 800 mg/kg BW) were able to increase GLUT4 expression, especially at 400 mg/kg BW. CTE2 was able to increase GLUT4 expression, nearly approached the NC. On the other hand, glibenclamide is an antihyperglycemic treatment that lowers blood glucose by downregulating IFNγ and improve pancreas. The decrease in blood glucose occurs through insulin secretion from pancreatic β-cells triggered by glibenclamide[23]. CTE 400 mg/kg BW was able to increase GLUT4 expression better than glibenclamide. These data enlarge findings regarding the potential of CTE to increase GLUT4 expression in type 2 DM.

5. Conclusion

Based on the results and discussion above, it is reported that CTE could rehabilitate the type 2 DM induced by STZ. The most effective CTE dose to reduce IFNγ expression is 400 mg/kg BW. In addition, the most significant CTE dose to increase GLUT4 expression is CTE 400 mg/kg BW as well.

6. Acknowledgements

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