RESEARCH ARTICLE

pISSN: 0126-074X | eISSN: 2338-6223
https://doi.org/10.15395/mkb.v58.4608
Majalah Kedokteran Bandung. 2026;58(2):138–146

Majalah Kedokteran Bandung (MKB)

Received: October 20, 2025
Accepted: February 19, 2026
Available online: June 30, 2026

# Modulatory Effects of *Clitoria ternatea* Extract on TNF-α Protein and *Gys* Gene Expression in Diabetic–Dyslipidemic Rats

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**Abstract**

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia resulting from impaired insulin secretion or insulin action. Dyslipidemia is a common complication of DM and is associated with an increased risk of cardiovascular disease. Although pharmacological therapies are widely used, their long-term use is often limited by adverse effects, highlighting the need for safer alternative therapeutic approaches. The butterfly pea (*Clitoria ternatea* L.) has been reported to possess antidiabetic and antioxidant properties; however, evidence regarding its effects on inflammatory mediators such as TNF-α and *Gys* gene expression in diabetic–dyslipidemic conditions remains limited. This study aimed to evaluate the modulatory effects of *C. ternatea* extract (CTE) on TNF-α expression and *Gys* gene expression in diabetic–dyslipidemic rats. This experimental study was conducted from July to September 2021 in Bogor and Bandung, Indonesia. Diabetic–dyslipidemic rats were attained by inducing streptozotocin (STZ) and a high fat diet (HFD). CTE was administered at doses of 200, 400, and 800 mg/kg body weight. Glibenclamide, simvastatin, and their combination were administered to diabetic rats as a comparison group. The immunohistochemistry assay was conducted to quantify liver TNF-α protein expression, and liver and muscle's *Gys* genes were determined by quantitative qRT-PCR. Results showed 800 mg/kg BW CTE had a positive effect on decreasing TNF-α expression and elevating liver and femoral muscle Gys expression. Butterfly pea extract has the potential as an antidiabetic by regulating TNF-α and *Gys* gene.

**Keywords:** Diabetes mellitus, dyslipidemia, glycogen, plant extracts, tumor necrosis factor

## Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both.[1] DM is categorized into two kinds based on the cause, which are type 1 and type 2. Type 1 arises from an autoimmune dysfunction that causes damage to cells in the pancreas through inflammation mediated by T and B immune cells; hence, the hormone insulin cannot be secreted normally. Type 1 DM results from autoimmune destruction of pancreatic β-cells mediated by T and B lymphocytes, leading to absolute insulin deficiency. In contrast, type 2 DM is characterized by insulin resistance, which is frequently associated with obesity and may eventually contribute to pancreatic β-cell dysfunction.[2] Dyslipidemia is a common complication of DM. High triglyceride (TG) levels and a decline in high density lipoprotein (HDL) cholesterol are the hallmarks of type 2 DM and dyslipidemia, alterations that are present long before clinically significant hyperglycemia.[3]

Chronic low-grade inflammation plays an important role in the pathogenesis of type 2 DM. Among the proinflammatory cytokines, particularly tumor necrosis factor-α (TNF-α), contributes to insulin resistance and disrupts glucose metabolism in type 2 DM. In type 2 DM, glycogen levels in peripheral tissues such as femoral muscle are also decreased due to a failure of glucose regulation. Glucose is a vital compound within the organism because it participates in the production of energy needed for daily

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activities and physiology. Glucose is stored in the form of glycogen by the body. The glycogen will be broken down back into glucose when the body needs energy. The body stores glycogen in the liver and femoral muscles.[4] However, the relationship between inflammation-associated TNF-α expression and glycogen especially the glycogen synthase (*Gys*) gene regulation in the liver and muscle under diabetic–dyslipidemic conditions has not been fully elucidated.

Various antidiabetic agents are available to control blood glucose levels; however, long-term use may be associated with adverse effects and inadequate control of inflammation-related metabolic complications. Consequently, increasing attention has been directed toward natural products as potential alternative therapeutic agentss. One such plant is butterfly pea (*Clitoria ternatea* L.), a perennial herb belonging to the Fabaceae family that is widely cultivated in tropical and subtropical regions.[5,6] In the medical field, *C. ternatea* extract (CTE) exhibits anti-inflammatory, diuretic, analgesic, nootropic, antipyretic, antilipidemic, antidiabetic, antioxidant, antiarthritis, and wound healing activities. These activities are attributed to the presence of flavonols and anthocyanins. Moreover, CTE was reported to possess various bioactive compounds, such as saponins, triterpenoids, phenols, and alkaloids.[7] In another study, CTE could reduce DM symptoms, such as high blood sugar, urea, creatinine, and glycosylated hemoglobin levels.[5] While CTE initially emerged in the South China Sea or Pacific Ocean, it has now proliferated throughout Australia, North America, South America, Asia, and Africa. This broad dispersion and prevalence of CTE could be utilized in potential applications in the medical sector.[8] Hence, the antidiabetic properties of CTE were examined in diabetic rats with dyslipidemia complications by evaluating several parameters, including TNF-α expression and *Gys* expression in the liver and femoral muscles. This study provides additional evidence on the potential of CTE in modulating inflammation and glucose metabolism, addressing the current lack of integrated evidence regarding its effects on TNF-α–associated inflammatory pathways and *Gys* gene expression in insulin-sensitive tissues under diabetic–dyslipidemic conditions. Therefore, this study aimed to evaluate the effects of CTE on TNF-α protein expression and *Gys* gene expression in the liver and femoral muscle of diabetic–dyslipidemic rats.

## Methods

This experimental study was conducted from July to September 2021 at IratCo Laboratory, Bogor, West Java, Indonesia, and the Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia. Ethical approval was obtained from the Ethics Committee of Maranatha Christian University, Indonesia (No. 147/KEP/VI/2021). The *Clitoria ternatea* extract (CTE) extract was supplied by PT FAST (Indonesia) with CoA No. Batch 00103211072. The extract was prepared using 70% ethanol and subsequently mixed with lactose to produce powdered CTE.[9,10]

Quality assessment of the extract was performed according to Regulation Number 32 of 2019 issued by the Indonesian Food and Drug Supervisory Agency, including sensory evaluation, physical characterization, and microbial contamination testing.

Thirty-two male Sprague-Dawley rats aged approximately 6 weeks and weighing 120–140 g were obtained from IratCo Laboratory, Bogor, Indonesia. The rats were randomly divided into eight experimental groups, with four rats per group (n=4). The acclimatization of rats was conducted for seven days in a cycle of 12 hours light/dark, with temperature of 25°C. A standard diet and *ad libitum water* were consumed by the rats.[11]

After acclimatization, dyslipidemia was induced by administering a high-fat diet (HFD) for 28 days consisting of 18% crude protein, 5.5% crude fiber, and 7.37% crude fat, supplemented with 0.01% propylthiouracil (PTU) (Dexa Medica).[12] Rats with dyslipidemia were confirmed by a total level of serum cholesterol ≥ 200 mg/dL by the Cholesterol Kit (Elabscsi, E-BC-K109-M). Serum cholesterol and glucose measurements were conducted solely as preliminary screening to confirm successful model induction and were not included as quantitative outcome variables in this study.

Diabetes mellitus was induced using nicotinamide (NA; Sigma-Aldrich N3376) and streptozotocin (STZ; Sigma-Aldrich S0130). Nicotinamide was dissolved in normal saline and administered intraperitoneally at 120 mg/kg body weight. Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5) and administered intraperitoneally at 60 mg/kg body weight 60 minutes after nicotinamide injection.[13] The rats were subsequently classified into 8 groups. Group I (NC: negative control) were rats that

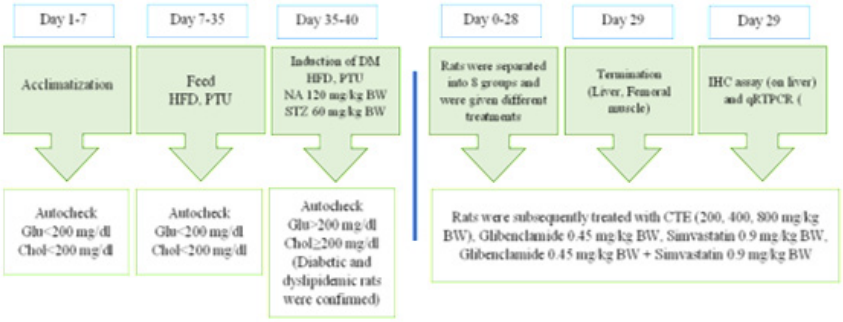


**Figure 1 Study Design Diagram**

were given distilled water 0.5 mL daily. Group II (PC: positive control) were STZ-induced rats that were fed with HFD. Group III were PC rats given with 200 mg/kg BW CTE. Group IV were PC rats given with 400 mg/kg BW CTE. Group V were PC rats receiving 800 mg/kg BW CTE. Moreover, Group VI was given glibenclamide (Generic, GKL9520905004A2) (0.45 mg/kg BW), group VII was given simvastatin (Generic, GKL131670271A) (0.9 mg/kg BW), while group VIII were administered both of them (glibenclamide and simvastatin).[9,13] The study design is presented in Figure 1

Following a 28-day course of CTE, 100 mg/kg BW of ketamine HCl from Ikapharmindo Putramas were used to anesthetized the rats. Xyla (Interchemie, 361453), administered at 10 mg/kg of BW, was used for euthanazation via intraperitoneal injection. Liver and femoral muscle tissues were subsequently collected.[11] A portion of these samples were well-kept-up at -80$^0$C, while the other samples were set in formalin (10%) for immunohistrochemistry (IHC) analysis.[10,11,14]

The liver was processed using the routine paraffin embedding method for immunohistochemistry assay. The tissue samples in the paraffin block were then deparaffinized using xylene 3 times for 3 min each. Then, slide preparations were rehydrated using 100%, 95% and 70% ethanol for 3 min, respectively. After 3 min, the slides were washed with water 1 min long and were incubated in a peroxidase block solution for 10 min. After that, the incubation for preparations was carried out for 10 min in prediluted blocking serum. The primary antibody or rabbit-anti-rat TNF-α (ElabScience, E-AB-40015) was put on and stored at the ambient temperature for 1 h. Haematoxyline is used for counterstained. An IHC kit developed specifically for rabbits, ab64261, was utilized to visualized the target protein utilizing HRP and DAB (ABC) detection. The Two-Step Plus Poly-HRP Anti Mouse/Rabbit IgG Detection System with DAB solution (Elabsci, E-IR-R217) was employed for protein target visualization.[10,11,14,15] TNF-α expression was quantified using ImageJ software from five randomly selected microscopic fields.

The RNA primer sequence shown in Table 1 that used for qRT-PCR assay. In this study, glycogen metabolism was represented by the analysis of the glycogen synthase (*Gys*) gene, a key enzyme responsible for glycogen synthesis in insulin-sensitive tissues. Direct-zol of RNA Miniprep Plus Kit (Zymo, R2073) was utilized to isolated then purified the total RNA from rat's liver and femoral muscle.

RNA concentration and purity values shown in Table 2 represent mean measurements obtained from samples in each experimental group. For

**Table 1 Primer Sequences Used for qRT-PCR Analysis**

| Genes | Primer sequence (5'-3')<br>F (Forward)<br>R (Reverse) | Product size (bp) | Annealing Temperature (°C) | NCBI Reference Sequence |
|---|---|---|---|---|
| Rat *Gys* | AGAGTTCGTCCGTGGCTGTC<br>GTTCGTGGAGATGCTCGGGA | 109 | 57 | NM_001109615.1 |
| Rat *Gapdh* | TCAAGATGGTGAAGCAG<br>ATGTAGGCCATGAGGTCCAC | 217 | 57 | NM_001289726 |

**Table 2 RNA Concentration and Purity**

| Samples | RNA Concentration (ng/µL) | RNA Purity (260/280 nm) |
|---|---|---|
| Negative Control | 2538.9 | 1.9193 |
| Positive Control | 2682.70 | 1.9031 |
| CTE1 (200 mg/kg BW) | 2262.70 | 1.8532 |
| CTE2 (400 mg/kg BW) | 1956.10 | 1.9432 |
| CTE3 (800 mg/kg BW) | 1882.70 | 2.0159 |
| Glibenclamide | 2168.60 | 1.8160 |
| Simvastatin | 1555.50 | 2.0442 |
| Glibenclamide +Simvastatin | 1759.20 | 1.9108 |

qRT-PCR, complementary DNA was synthesized by iScript Reverse Transcription Supermix (Bio-Rad, 170-8841). This quantitative analysis of gene expression carried out by AriaMx 3000 Real-Time PCR System (Agilent). The reaction mixture for qRT-PCR was the Evagreen master mix.[10,14,16]

Data were expressed as mean ± standard deviation. Statistical analysis was performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical significance was set at $p<0.05$

**Table 3 CTE specification analysis**

| Item | Specification | Method | Result |
|---|---|---|---|
| **Organoleptic** | | | |
| Form | Powder | Internal | Accordance |
| Color | Dark Blue | Internal | Accordance |
| Odor | Typical | Internal | Accordance |
| Flavor | Slightly Sour | Internal | Accordance |
| **Physical Characteristic** | | | |
| Extract ratio | 1:1 | Internal | Accordance |
| 80 mesh testing | ≥ 90% pased | Internal | Accordance |
| Solubility | Salution in water | Internal | Accordance |
| Water content | < 10% | 432/01/2019/QC | Accordance |
| Heavy Metal contamination Lead (Pb) | ≤10 ppm | External Laboratory | 0,0008 |
| Cadmium (Cd) | ≤0,3 ppm | | 0,002 |
| Arsenic (As) | ≤5 ppm | | 0,008 |
| Mercury (Hg) | ≤0,5 ppm | | 0,0007 |
| **Microbiological contamination** | | | |
| Total plate number (ALT) | $\leq 10^5$ | External Laboratory | $1,5 \times 10^2$ |
| Yeast mold number (AKK) | $\leq 10^5$ | | $9,0 \times 10^1$ |
| *E. Coli* | $\leq 10^1$ | | $1,0 \times 10^1$ |
| *Enterobacteriaceae* numbers | $\leq 10^5$ | | $<1,0 \times 10^1$ |
| *Clostridia* | Negative | | Negative |
| *Salmonella* | Negative | | Negative |
| *Shigella* | Negative | | Negative |

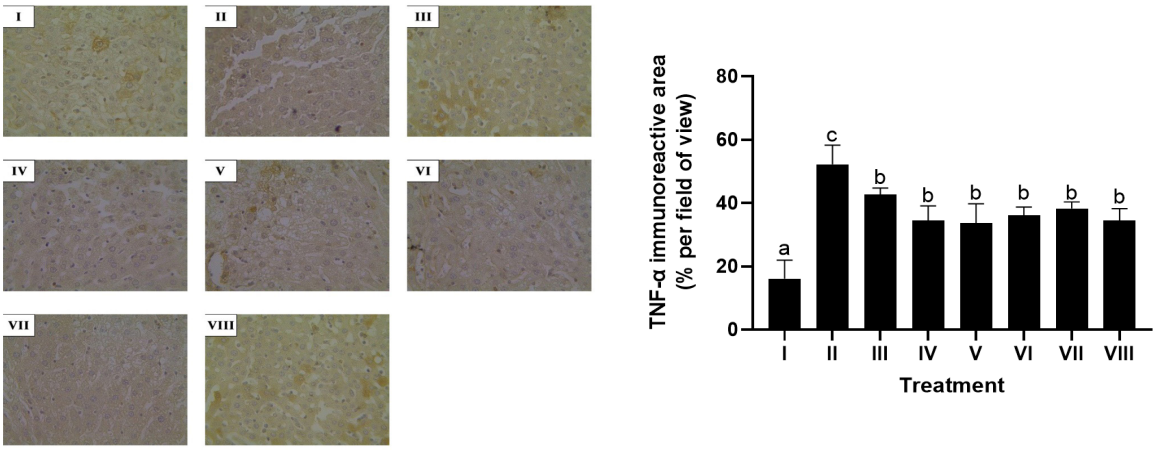


**Figure 2 Effect of *Clitoria ternatea* Extract on Hepatic TNF-α Protein Expression in Diabetic–Dyslipidemic Rats**

(A) Representative immunohistochemical staining of TNF-α expression in liver tissue. (B) Quantitative analysis of TNF-α immunoreactivity using ImageJ software. Group I = Negative Control (NC); Group II = Positive Control (PC); Group III = PC + Clitoria ternatea extract (CTE) 200 mg/kg body weight (BW); Group IV = PC + CTE 400 mg/kg BW; Group V = PC + CTE 800 mg/kg BW; Group VI = PC + Glibenclamide 0.45 mg/kg BW; Group VII = PC + Simvastatin 0.9 mg/kg BW; Group VIII = PC + Glibenclamide 0.45 mg/kg BW + Simvastatin 0.9 mg/kg BW. Data are presented as mean ± standard deviation (SD). Different superscript letters indicate statistically significant differences among groups ($p<0.05$, one-way analysis of variance [ANOVA] followed by Tukey's post hoc test

## Results

The quality assessment of Clitoria ternatea extract (CTE), including organoleptic characteristics, physical properties, heavy metal contamination, and microbiological contamination, complied with the requirements of the Indonesian Food and Drug Supervisory Agency Regulation Number 32 of 2019. The detailed quality assessment results are presented in Table 3.

All rats subjected to high-fat diet and *streptozotocin* induction met the predefined criteria for diabetic–dyslipidemic status based on preliminary serum cholesterol and glucose screening prior to treatment. TNF-α protein expression in liver tissue is presented in Figure 2. Immunohistochemistry (IHC) is a procedure used to detect antigens within cells of a tissue sample by employing antibodies that bind specifically to those antigens in biological tissues, in order to determine the pathology of a tissue. According to the result, the positive control group (DM rats) exhibited robust staining in the cytoplasm, indicating elevated levels of TNF-α expression. Groups III – V (CTE treatment at 200, 400, 800 mg/kg BW) decreased TNF-α expression in contrast to the positive control ($p<0.05$) (Figure 2B). Diabetic rats model exhibited significantly higher liver TNF-α expression in contrast to the negative control ($p<0.05$). Groups III – V notably decreased liver TNF-α expression contrary to diabetic model rats. The most efficient dose of CTE to reduce TNF-α expression was Group V (CTE 800 mg/kg BW). This result outlines the CTE's capacity to control TNF-α's expression, leading to its reduction.

Values are presented as mean ± SD. Different superscript letters indicate statistically significant differences among groups ($p<0.05$, one-way ANOVA followed by Tukey's post hoc test).

The effects of CTE on Gys gene expression in liver tissue are shown in Figure 3A. All CTE-treated groups (Groups III–V) demonstrated higher Gys gene expression than the positive control groupGroup V (800 mg/kg BW) attained the highest value and the most effective to increased liver *Gys* on diabetes model rats. Group V (CTE 800 mg/kg BW) portrayed the most significant value in increasing *Gys* gene expression, even higher than the treatment of glibenclamide, simvastatin, and both of them. This supports the hypothesis, namely that the administration of CTE increases the expression of glycogen genes in DM rats' liver. Similar to its storage in the liver, *Gys* is also accumulated in

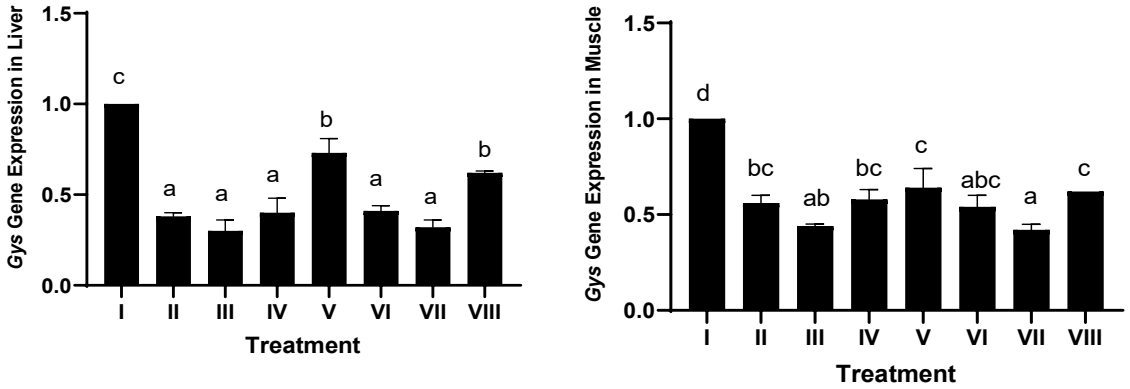


**Figure 3 Effect of *Clitoria ternatea* Extract on *Gys* Gene Expression in Liver (A) and Femoral Muscle (B) Tissues of Diabetic–Dyslipidemic Rat**

Groups: I = Negative Control (NC); II = Positive Control (PC); III = PC + CTE 200 mg/kg BW; IV = PC + CTE 400 mg/kg BW; V = PC + CTE 800 mg/kg BW; VI = PC + Glibenclamide 0.45 mg/kg BW; VII = PC + Simvastatin 0.9 mg/kg BW; VIII = PC + Glibenclamide 0.45 mg/kg BW + Simvastatin 0.9 mg/kg BW. Values are presented as mean ± SD. Different superscript letters indicate statistically significant differences among groups ($p<0.05$, one-way ANOVA followed by Tukey's post hoc test)

muscle tissue. Decreased glycogen is found in patient with any type of DM. The results of *Gys* gene expression in muscles by qRT-PCR can be seen in Figure 3B. Groups III, IV, and V (CTE 200, 400, and 800 mg/kg BW) were able to increase *Gys* gene expression in the femoral muscle contrary to the positive control. In addition, Group V (CTE 800 mg/kg BW) displayed the most notable rise in *Gys* gene expression, even better than glibenclamide (Group VI) and simvastatin treatments (Group VII). This result illustrates the ability of CTE in regulate diabetes mellitus by elevating the level of the *Gys* gene.

## Discussion

The primary objective of this study was to evaluate the antidiabetic activity of CTE in diabetic rats with diabetic-associated complications. Insulin plays a central role in glucose homeostasis, and impairment of insulin signaling is a key feature of diabetes mellitus. Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine predominantly produced by macrophages and adipocytes, contributes to insulin resistance by disrupting insulin signaling pathways and impairing glucose metabolism[17] In the present study, CTE administration reduced hepatic TNF-α expression in diabetic–dyslipidemic rats, with the greatest reduction observed at a dose of 800 mg/kg body weight. These findings suggest that CTE may attenuate inflammation-associated insulin resistance through modulation of TNF-α expression..

Insulin resistance ensued due to the impact of TNF-α, as it plays a pivotal inflammation role through the reactive oxygen species (ROS) generation, and it is synthesized by adipocytes and peripheral tissues. In insulin resistance, this factor can interfere with insulin signaling through serine phosphorylation.[18] TNF-α, a proinflammatory cytokine, involved in T2DM by the skeletal and cardiac muscles as well as adipocytes have the glucose transporter type 4 (GLUT4). Through serine phosphorylation pathway of insulin receptor substrate-1, TNF-α prevents the insulin effectivity. Consequently, this process causes insulin resistance in peripheral tissues.[19]

In the present study, CTE treatment reduced TNF-α expression compared with the positive control group, with the greatest reduction observed at a dose of 800 mg/kg body weight. This finding suggests that CTE may alleviate inflammation-associated insulin resistance through suppression of TNF-α expression. The observed effect is consistent with previous studies demonstrating that anthocyanins and other polyphenolic compounds present in CTE exhibit anti-inflammatory activity by inhibiting the IKK/NF-κB signaling pathway, leading to reduced production of proinflammatory cytokines such as TNF-α and IL-6.[20]

The liver utilizes stored glycogen to control blood glucose levels. The presence of hepatic

insulin resistance increases endogenous *Gys* production (Figure 3A). CTE increased *Gys* gene expression mainly by treatment with 800 mg/kg BW. This result was verified by previous research reporting that CTE could trigger a glycogen rise in people with DM. Patients with DM will lack insulin or have insulin retention. Patients of type 2 DM with hyperlipidemia and hyperinsulinemia have decreased glycogen in the liver and muscle due to declined insulin signaling. When insulin function is impaired, it hinders glucose metabolism and diminishes glycogen levels.[21] Previous studies have indicated that glycogen, although present in limited amounts, can be detected in adipose tissue under insulin-resistant and inflammatory conditions, reflecting altered glucose metabolism.[22] Furthermore, diabetes mellitus is noted in influencing structural alterations in glycogen. It was postulated that the heightened fragility of α particles in mice with T2DM may contribute to poor glucose regulation. This is because smaller glycogen molecules degrade rapidly.[23] In the present study, Gys gene expression in femoral muscle was similarly increased following CTE administration, particularly at the dose of 800 mg/kg body weight. This observation supports previous evidence showing that plant extracts rich in flavonoids, anthocyanins, and saponins can enhance glycogen metabolism and improve insulin sensitivity in peripheral tissues.[24]

The observed improvement in hepatic and muscular *Gys* expression, accompanied by decreased TNF-α levels, supports the dual antioxidant and anti-inflammatory mechanisms of CTE. This is consistent with the findings of Johan *et al*. and Philip *et al*., who demonstrated its α-amylase inhibition and radical scavenging activities.[25,26] Previous studies also reported that CTE reduced diabetes-related symptoms, including elevated blood glucose, urea, creatinine, and glycosylated hemoglobin levels,[6] attributed to its rich polyphenol content. In this study, induction of STZ combined with a high-fat diet successfully triggered hyperglycemia and dyslipidemia, as shown by depleted glycogen levels in both femoral muscle and liver, and elevated TNF-α expression. Administration of CTE restored glycogen levels in those tissues while simultaneously reducing TNF-α expression. Overall, these findings summarize the potential of CTE as an antidiabetic agent with dual regulatory effects on inflammation and glucose metabolism.

The limitation of this study is the absence of quantitative analysis of serum glucose and lipid parameters as outcome measures, as these parameters were used solely to confirm successful establishment of the diabetic–dyslipidemic model before treatment. Further studies are needed to elucidate the underlying molecular mechanisms and to evaluate the efficacy and safety of Clitoria ternatea extract in clinical settings.

In conclusion, *Clitoria ternatea* extract demonstrated beneficial antidiabetic effects by reducingTNF-α expression and increasing *Gys* gene expression in both liver and femoral muscle tissues of diabetic model rats. The 800 mg/kg BW dose showed the most pronounced activity, indicating CTE's strong potential in modulating inflammation and glucose metabolism related to diabetes mellitus. notably, the effects observed at this dose were comparable to or greater than those achieved by standard antidiabetic and lipid-lowering therapies, including glibenclamide (Group VI), simvastatin (Group VII), and their combination (Group VIII), particularly in reducing TNF-α expression and enhancing *Gys* gene expression.

## Acknowledgment

The authors sincerely appreciating Maranatha Christian University, Bandung, Indonesia, for Internal Funding as a monetary aid under grant Number 034/SK/ADD/UKM/VI/2021. This study also - supported by Aretha Medika Utama (AMUBBRC), Bandung, Indonesia.

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