

## Potential of *Clitoria ternatea* L. Extract Towards Insulin Receptor Expression and Marker of Inflammation in Diabetes Mellitus Rats Model

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### ABSTRACT

Metabolic abnormalities caused by the accumulation of human, environmental, genetic and lifestyle variables can be found in diabetes mellitus (DM). An increase in blood glucose carried on by a reduction in insulin production can also result in DM. Insulin resistance often occurs as a result of obesity and a lack of physical activity and aging. Telang flower extract (*Clitoria ternatea* L.) is reported to have several qualities such as being able to treat DM, chronic bronchitis, goiter, mucosal disorders and leprosy. This study's objective was to elucidate anti-dibetic effect of *C. ternatea* extract (CTE) in rats with diabetic complications related to dyslipidemia. Rats suffering from diabetes will be examined after being given oral doses of Simvastatin 0.9 mg/kg BW, Glibenclamide 0.45 mg/kg BW, and 200, 400, and 800 mg/kg BW CTE, model induced by streptozotocin. The expression of the insulin gene (INS-1) was investigated by qRT-PCR, Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ), and Interleukin-1 $\beta$  (IL-1 $\beta$ ) pancreatic DM rats model using the Immunohistochemistry (IHC) test. Both descriptive and quantitative data were acquired for the data. After utilizing ANOVA to evaluate quantitative data, the Tukey post hoc test was used to analyze the data, the expression of TNF- $\alpha$  and IL-1 $\beta$  was found to decrease while INS-1 expression increased in response to CTE. This effect was attributed to the modulation of TNF- $\alpha$ , IL-1 $\beta$ , and INS-1 expression. These findings suggest that CTE possesses antidiabetic properties.

## 1. Introduction

A chronic disorder known as diabetes mellitus (DM) causes blood glucose levels to rise as a result of the body's inability to generate insulin, producing insufficient amounts of it, or having trouble using it (Sadikot *et al.* 2017). In 2019, 9.3% or around 463 millions of people globally have experienced diabetes and this will increase in 2030 to 10.2%, affecting an estimated 578 million individuals. This number will continue to increase until 2045 to 700 million people, or 10.9% (Saeedi *et al.* 2019). In Indonesia, the prevalence for people who are not diagnosed with DM is around 4.1% of the total 5.6% of the population with diabetes (Pramono *et al.* 2010). According to medical diagnoses, 1.5% of

inhabitants under the age of 15 had diabetes in 2013, and by 2018, that number had risen to 2% (Ligita *et al.* 2019).

Insulin resistance is the most common flaw in those who have metabolic syndrome or are overweight. Insulin sensitivity and energy expenditure are both influenced by adipose tissue (Abdel *et al.* 2020). Decreases in adiponectin levels and increases in tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), free fatty acids, and resistant levels are signs of insulin-mediated disruption of glucose absorption brought on by insulin resistance (Esterson *et al.* 2013; Abdel *et al.* 2020). The insulin receptor (InsR), which is a membrane-covering glycoprotein, functions as a regulator of insulin. activation of a number of intracellular pathways results in increased glycoprotein and glucose absorption as well as a decrease in hepatic/muscle glucose output due to insulin binding to InsR in the

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liver, muscle or adipose tissue. As a result, blood glucose levels will decrease (Zhang *et al.* 2010; Abdel *et al.* 2020).

Chemical pharmaceuticals are known to cause several adverse effects that might affect patients in the long term. Insulin injections and commonly prescribed drugs such as metformin and pioglitazone produce adverse symptoms such as dyspepsia, hypoglycemia, and insulin resistance (Liu *et al.* 2016). Hence, herbal medicine emerges as a viable solution harnessing the beneficial properties of its constituent compounds, such as those found in the telang flower. Telang flower (*Clitoria ternatea* L.) has a number of active ingredients, including tannins, anthocyanins, alkaloids, flavonoids, saponins, phenolic acids, kaempferol, maldivin, delphinidin, rutin, and quercetin (Gupta *et al.* 2010; Kalyan *et al.* 2011; Verma *et al.* 2013). These compounds have pharmacological activities such as antidiabetic and antioxidant (Kavitha 2018). Therefore, this study used a DM rats model to examine the antidiabetic effects of *C. ternatea* L. ethanolic extract (CTE).

## 2. Materials and Methods

### 2.1. Animals

In experiments using test animals, this study used male Sprague Dawley rats weighing around 120-140 g at 6 weeks of age. Rats were cared for in a natural cycle of light and darkness for 12 hours with a warm room temperature of 25°C. The acclimatization is carried out for 7 days regularly feed and water is given in unlimited quantities. Animal testing has been granted permission by the Ethics Committee of Maranatha Christian University (Ref no 147/KEP/VI/2021).

### 2.2. Extract Preparation

The plant in the form of telang flower (*C. ternatea* L.) was found in from Indonesia, precisely in East Java province of Pasuruan City, Prigen Regency, Sukoliko Village, which is precisely in the Herbal Village. The flower collection is carried out by the extraction process at PT FAST Traditional Medicine Industry (IOT), which is standardized by Good Manufacturing Practice (GMP). Maceration technique is used as an extraction process, employing 70% ethanol as a solvent and lactose as an additional ingredient to produce telang flower powder extract. Butterfly flower powder that has received a certificate of analysis (Batch Number: 00103211072) is stored at

room temperature. A stock solution is made which is diluted with distilled water and stored at -20°C for further *in vivo* testing (Widowati *et al.* 2018a, 2023).

### 2.3. STZ Induction of DM Rats Model

For 28 days, DM rats model were given a high-fat diet (HFD) together with propylthiouracil 0.01% (PTU, Dexa Medica) in Aquades (Kurniati *et al.* 2021). Streptozotocin (STZ, Sigma Aldrich SO130) was injected intraperitoneally to induce DM with dose 60 mg/kg BW. Aquades were given via the same route to the negative control group. Autocheck was used to measure the blood sugar and cholesterol levels of fasting rats five days later (Elamin *et al.* 2018). Rats with STZ-induced hypercholesterolemia (HCT) and fasting blood glucose (FBG) >250 mg/dl were employed in this investigation since they were classified as diabetic (Widowati *et al.* 2023).

### 2.4. CTE Treatment in DM Rats Model

Eight groups, each comprising four rats, were formed by randomly partitioning the DM rats model. Rats in Group I, the negative control (NC), were given 0.5 cc of distilled water once daily. Rats with STZ induction in Group II (positive control) were fed HFD and also given 0.5 cc of distilled water treatment. Group III, IV, and V were given the CTE with doses of 200, 400, and 800 mg/kg BW, in turn. Group VI (Glibenclamide 0.45 mg/kg BW), in group VII were received simvastatin (0.9 mg/kg BW), Group VIII included rats who got both of them (glibenclamide and simvastatin). Each group got treatment for 28 days. Immunohistochemistry (IHC) evaluation was performed after pancreatic tissue was preserved in 10% formalin. Pancreatic tissue was taken after euthanasia via cervical dislocation. On day 28 after CTE treatment, blood were taken from retro-orbitalis in 12 h-fasted rats before anesthesia (Widowati *et al.* 2023).

### 2.5. Quantification of INS-1 Gene Expression

Direct-zol RNA Miniprep Plus Kit (Zymo, R2073) to extract and purify rat is used as a tool to cool total RNA from pancreatic rats through extraction and purification, according to the manufacturer's instructions (Zymo, R2073). Applying iScript Reverse Transcription Supermix for RT-PCR (Bio-Rad, 170-8841), complementary DNA synthesis was carried out. The AriaMx 3000 Real-Time PCR System (Agilent G8830A) used to measure quantitative gene expression. The Evagreen master mix (Bio-

Rad, 1725200) was reaction mixture used for the qRT-PCR. The cycle used in the qPCR procedure is starting with denaturation for 30 seconds, carrying out 40 cycles at a temperature of 95°C, followed by annealing for 40 seconds at a temperature of 60°C and ending with an elongation process for 60 seconds at a temperature of 72°C. The pre-denaturation step took place for 7 min at 95°C. Using the Delta-Delta-Ct (ddCt) Algorithm, approach, qRT-PCR, gene expression, a relative copy number was used, in comparison to control (Widowati *et al.* 2018b, 2019; Afifah *et al.* 2019; Lucianus *et al.* 2024; Darsono *et al.* 2024). Table 1 lists the glycogen primer sequences.

## 2.6. Immunohistochemistry Assay

For IHC staining, 4 µm-thick paraffin blocks were cut. IHC staining with anti-TNF-α/IL-1β primary antibody (TNF-α, Elabscience E-AB-33121; IL-1β, Elabscience E-AB-33471). IHC staining with secondary antibody anti TNF-α/IL-1 antibody using polyclonal antibody 1:1,000 conjugated in HRP). Two-step plus Poly-HRP Anti Mouse/Rabbit IgG DetectionSystem (with DAB solution) (Elabsci, E-IR-R217) was used for visualizing the protein target The HRP/DAB (ABC) rabbit specific detection IHC kit was used to visualize the protein target The HRP/DAB (ABC) rabbit specific detection IHC kit was used to visualize the protein target (Abcam, ab64261) (Widowati *et al.* 2023; Onggowidjaja *et al.*, 2024; Darsono *et al.* 2024; Lucianus *et al.* 2024).

## 2.7. Statistical Analysis

All the data's mean and standard deviation are shown. Using the Tukey post Hoc test analyzing the data, and the One Way ANOVA test (P<0.05) indicated the result are meaning have a different significant.

Table 1. Primer sequence

Gene	Primer Sequence (5' to 3')	Annealing (°C)	Cycle	Reference
INS-1	AAC AGC ACC TTT	57	40	www.ncbi.nlm.nih.gov
	GTG GTT CTC			
GADPH	CAG TTG TGC	57	40	www.ncbi.nlm.nih.gov
	CAC TTG TGG G			
GADPH	TCAAGATGGTGAA	57	40	www.ncbi.nlm.nih.gov
	GCAG			
	ATGTAGGCCATGA			
	GGTCCAC			

## 3. Results

### 3.1. Glucose Levels on Diabetic Rats Model

CTE results on glucose levels of DM rats on day 28 are shown in the Table 2. Positive control (PC) with inducing, STZ, HFD and PTU increased rats blood glucose levels increased significantly compared to negative controls (NC, untreated rats). Treatment with CTE decreased serum glucose levels in a DM rats model. The most effective in reducing serum glucose levels is CTE 400 mg/kg BW.

### 3.2. INS-1 Gene Expression

Figure 1 showed that CTE 200-800 mg/kg BW significantly increased (P<0.05) INS-1 gene expression in DM rats model. The INS-1 relative gene expression of group V (CTE 800 mg/kg BW) was the most active to increase INS-1 gene expression in DM rats model.

### 3.3. IL-1β Expression

The IL-1β protein is shown in this study by a brown hue in positive control. The pancreatic IL-1β expression of NC, less brown hue can be seen in Figure 2. The intensity of the brown hue (light brown) was less in the treatment at 200 mg/kg BW, suggesting that the CTE may have downregulated significantly (P<0.05) IL-1β expression in the pancreas. The deeper color may be understood as the greater the expression of IL-1β.

Figure 3 shows that CTE decreased the (41.88±4.91%). The lowest IL-1β expression was seen with 400 mg/kg BW of CTE (26.42±3.98%), compared to a doses 800 and 200 mg/kg BW which had IL-1β expression of 38.01±4.63% per view and 37.24±4.15% per view respectively. This results indicates that CTE

Table 2. The effect of CTE towards glucose levels in DM rats model

Group	Glucose levels (mg/dl)
I	92.63±2.28 <sup>a</sup>
II	399.49±34.57 <sup>g</sup>
III	353.24±9.36 <sup>f</sup>
IV	229.86±2.57 <sup>c</sup>
V	303.70±9.77 <sup>e</sup>
VI	179.12±0.88 <sup>b</sup>
VII	272.52±1.15 <sup>d</sup>
VIII	184.25±5.56 <sup>b</sup>

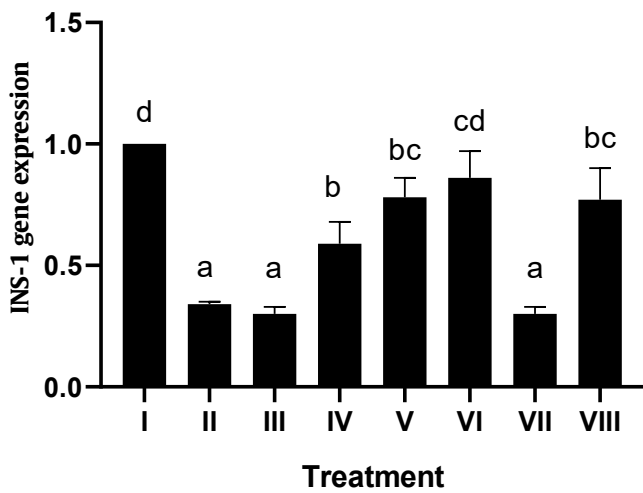


Figure 1. The effect of CTE towards INS-1 gene expression in DM rats model

\*Mean ± standard deviation is shown as data. Note: I: (negative control), II: (PC), III: (PC + 200 mg CTE/kg BW) (CTE1), IV: (PC + 400 mg CTE/kg BW) (CTE2), V: (PC + 800 CTE mg/kg BW) (CTE3), VI: (PC + 0.45 mg glibenclamide/kg BW), VII: (PC + 0.9 mg simvastatin/kg BW), VIII: (PC + 0.45 mg glibenclamide /kg BW and 0.9 mg simvastatin/kg BW) Based on Tukey's HSD post-hoc test ( $p < 0.05$ ), various letters (a, b, bc, cd, d) show significant differences among treatments

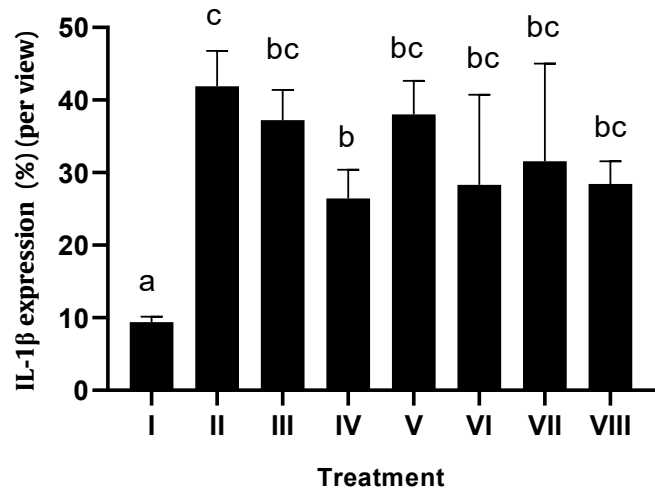


Figure 3. The effect of CTE towards IL-1β protein expression in DM rats model

\*Mean ± standard deviation is shown as data. Note: I: (negative control), II: (PC), III: (PC + 200 mg CTE/kg BW) (CTE1), IV: (PC + 400 mg CTE/kg BW) (CTE2), V: (PC + 800 CTE mg/kg BW) (CTE3), VI: (PC + 0.45 mg glibenclamide/kg BW), VII: (PC + 0.9 mg simvastatin/kg BW), VIII: (PC + 0.45 mg glibenclamide /kg BW and 0.9 mg simvastatin/kg BW) Based on Tukey's HSD post-hoc test ( $p < 0.05$ ), various letters (a, b, bc, c) show significant differences among treatments

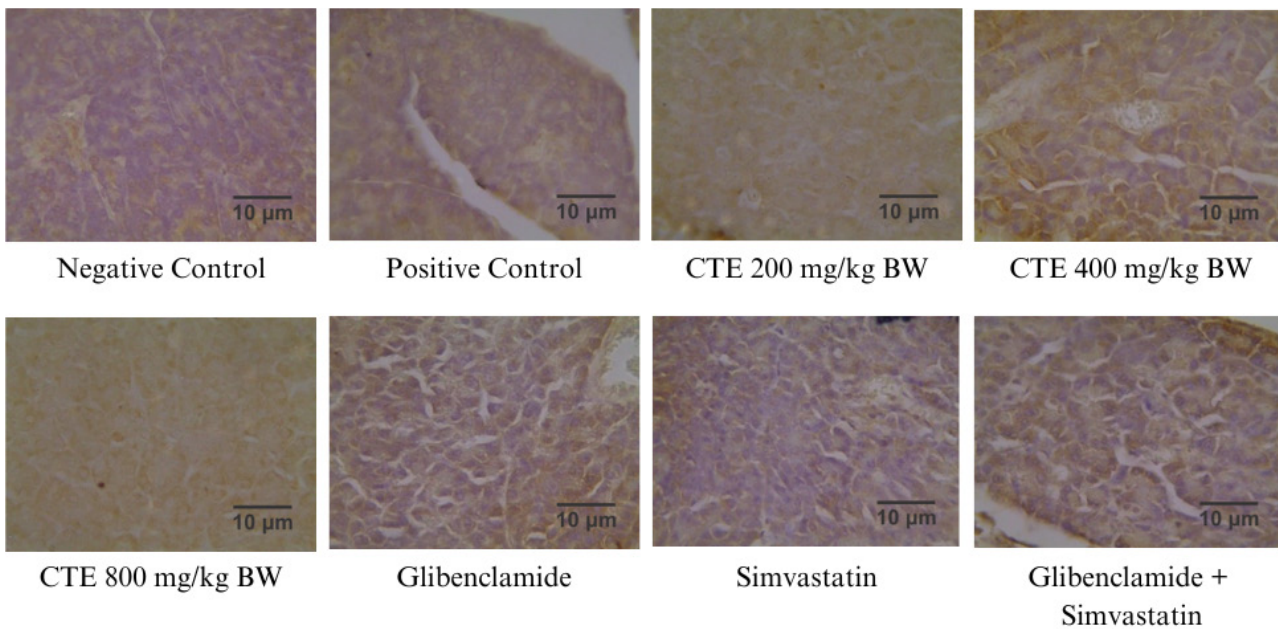


Figure 2. IHC staining of IL-1β expression in the pancreas of DM rats model. I: (negative control), II: (PC), III: (PC + 200 mg CTE/kg BW) (CTE1), IV: (PC + 400 mg CTE/kg BW) (CTE2), V: (PC + 800 CTE mg/kg BW) (CTE3), VI: (PC + 0.45 mg glibenclamide/kg BW), VII: (PC + 0.9 mg simvastatin/kg BW), VIII: (PC + 0.45 mg glibenclamide /kg BW and 0.9 mg simvastatin/kg BW) (magnification ×40)



treatment has anti-inflammatory properties that affect the inflammatory inhibition in DM rats model.

### 3.4. TNF- $\alpha$ Expression

Brown highlights indicate the presence of TNF- $\alpha$  protein. The negative control cells (Figure 4) do not display any such color. The reduction in brown hue intensity (light brown) at 200, 400, 800 mg/kg BW suggests that CTE may have the ability to significantly downregulate ( $P < 0.05$ ) TNF- $\alpha$  protein expression. The pancreatic TNF- $\beta$  expression positively stained with darker brown color.

Figure 5 shows that the treatment of CTE reduced the TNF- $\alpha$  compare to positive control ( $59.68 \pm 6.59\%$ ). CTE at all doses (200, 400, 800 kg/BW), glibenclamide, simvastatin, combination of glibenclamide and simvastatin have similar antiinflammatory potent to decrease TNF- $\alpha$  protein expression in DM rats model.

### 4. Discussion

In rats with DM complications caused by dyslipidemia, this study's objective was to evaluate CTE as anti-DM activity. Numerous active chemicals,

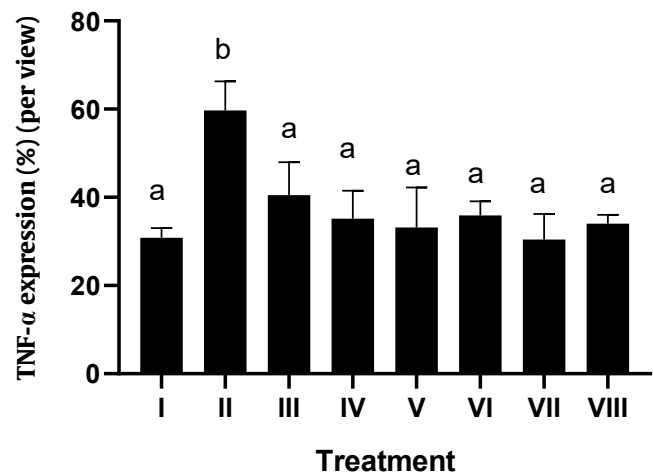


Figure 5. The effect of CTE towards TNF- $\alpha$  protein expression in DM rats model

\*Mean  $\pm$  standard deviation is shown as data. I: (negative control), II: (PC), III: (PC + 200 mg CTE/kg BW) (CTE1), IV: (PC + 400 mg CTE/kg BW) (CTE2), V: (PC + 800 CTE mg/kg BW) (CTE3), VI: (PC + 0.45 mg glibenclamide/kg BW), VII: (PC + 0.9 mg simvastatin/kg BW), VIII: (PC + 0.45 mg glibenclamide /kg BW and 0.9 mg simvastatin/kg BW) Based on Tukey's HSD post-hoc test ( $p < 0.05$ ), various letters (a, b) show significant differences among treatments

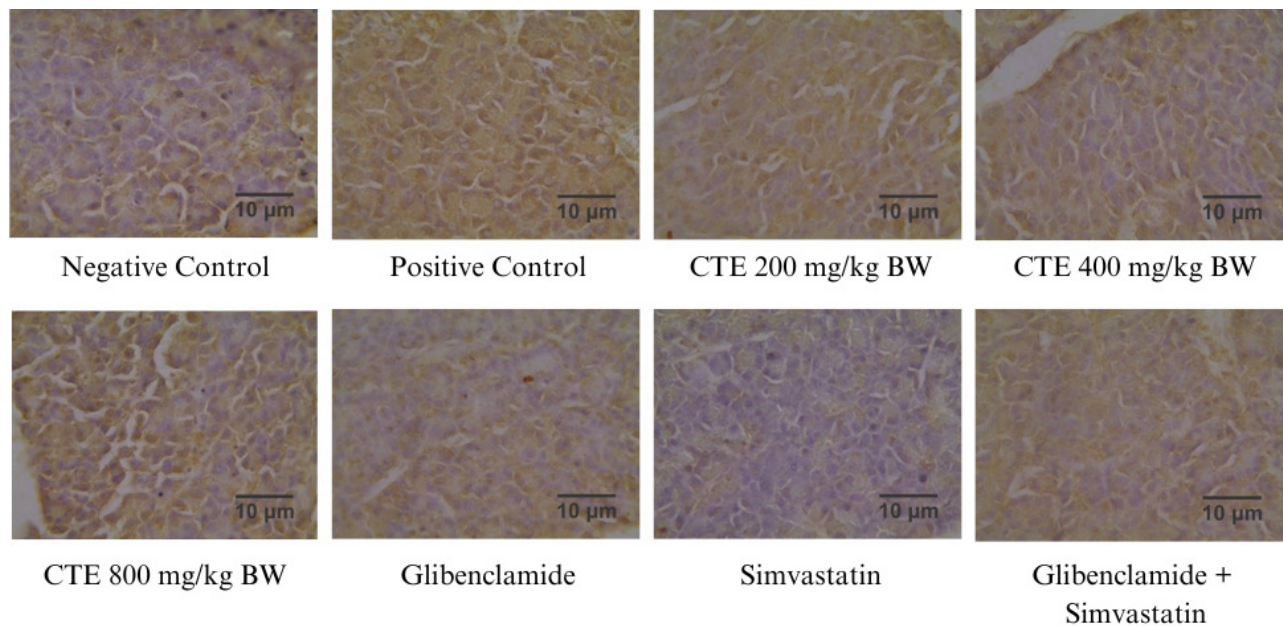


Figure 4. IHC staining TNF- $\alpha$  expression in the pancreas of DM rats model. I: (negative control), II: (PC), III: (PC + 200 mg CTE/kg BW) (CTE1), IV: (PC + 400 mg CTE/kg BW) (CTE2), V: (PC + 800 CTE mg/kg BW) (CTE3), VI: (PC + 0.45 mg glibenclamide/kg BW), VII: (PC + 0.9 mg simvastatin/kg BW), VIII: (PC + 0.45 mg glibenclamide /kg BW and 0.9 mg simvastatin/kg BW) (magnification  $\times 40$ )

including 2-Hydroxycinnamate, pyruvic acid, Inositol, Catechin-7-O-glucoside, and Delphinidin 3-(6-p-coumaroyl) glucoside were found in CTE in before investigation (Widowati *et al.* 2023). These active compounds have potential as anti-DM. Insulin plays a vital role in DM. When a patient has DM, insulin cannot be produced, thereby increasing the glucose levels in the blood. The  $\beta$ -cells of the pancreas produce insulin. In rats, there are two signalling genes in insulin formation, namely INS-1, and INS-2. The difference lies in the chromosomes but more or less have the same function, namely, encoding the formation of insulin (Babaya *et al.* 2006). STZ promotes fat and DM by damaging pancreatic  $\beta$ -cells and inhibiting insulin production and release (Talpatte *et al.* 2013). Oxidative stress induced by ROS is a mechanism that causes disruption of insulin signaling and reduces insulin-stimulated insulin-responsive cell types' absorption of glucose in target tissues, including skeletal muscle, the liver, and the adipose.

In our study, HFD, PTU, and STZ induction in rats cause rats to develop DM with blood glucose levels  $\geq 250$  mg/dl (Table 2). This is consistent with previous research that show STZ, PTU and HFD can increase blood sugar levels in DM rat models (Arafa *et al.* 2016). Blood glucose levels can be reduced by CTE in DM rats model. This can be influenced by the content of 2-hydroxycinnamic acid in CTE it has been confirmed to have an antidiabetic impact by reducing blood glucose in wistar rats (Ambika *et al.* 2013).

CTE can increase the expression of the INS-1 gene in the pancreas of DM rats model (Figure 1). This is consistent with other research showing that CTE can boost insulin and lower blood glucose levels in diabetic rats caused by STZ (Kavitha *et al.* 2018). Based on another study, in STZ-induced DM model rats, CTE demonstrated pancreatic regeneration, suggesting its effectiveness as an antidiabetic (Verma *et al.* 2013). The phenolics content in CTE have biological activities and can be used the medicinal effects such as antibacterial, antioxidant, and antidiabetic (Indrianingsih *et al.* 2021).

IL-1 $\beta$  which is the proinflammatory cytokine and an increasing of IL-1 $\beta$  can cause damage to pancreatic  $\beta$  cells, decreased insulin production and also leads to other inflammation (Godfrey *et al.* 2020). In the process of  $\beta$  cells death, Protein kinases that are triggered by Mitogen Activated Protein Kinase (MPAK) and Nuclear Factor-kappa B are largely

affected by IL-1 $\beta$  (Liu *et al.* 2016). In this study, CTE can suppress IL-1 $\beta$  expression that marked in less brown colour based on IHC staining in pancreas of diabetic rats (Figure 2). CTE lowered the glucose level and increased the body weight compared to glibenclamide, indicated CTE has antihyperglycemic impact in rats with DM caused by alloxan (Rajamanickam *et al.* 2015).

The pancreas' link between TNF- $\alpha$  and IL-1 $\beta$  is that TNF- $\alpha$  may cause intraislet resident macrophages to release IL-1 $\beta$ , which causes iNOS expression and excessive NO generation in  $\beta$ -cells (Atkinson *et al.* 2011). The ability of CTE to act as an antidiabetic was also shown by reducing the intensity of the brown color that express of TNF- $\alpha$  protein in the IHC results (Figure 4). Recent research indicates that when the pancreas is under stress, it synthesizes TNF- $\alpha$ , and with the islets being mainly responsible for this production (El Nawawy *et al.* 1998).

The prior research found that *C. ternatea's* anthocyanins inhibit the production and activity of pro-inflammatory chemicals such IL-6, TNF- $\alpha$ , NF- $\kappa$ B, IL-1 $\beta$ , and Cyclooxygenase-2 (COX-2) providing anti-inflammatory benefits (Yanti *et al.* 2020). This matches the findings of the performed research. Consisting of proteins, Volatile oils, alkaloids, stigmastane-3,6-dione, anthocyanins, cardiac glycosides, steroids, anthraquinone, saponins, triterpenoids, phenols, flavanoids, and flavonol glycosides (Al-Snafi 2016). CTE has antidiabetic and antioxidant activity due to its polyphenol compound (Indrianingsih *et al.* 2021). Various polyphenols included in foods and supplements can increase resistance by modifying insulin signaling pathways, lowering postprandial glucose, and avoiding damage to pancreatic cells that release insulin (Williamson and Sheedy 2020). Flavonoids act as hypoglycemic agents (Ghorbani 2017). The potential of flavonoids to lower glucose absorption or enhance glucose tolerance is connected to their hypoglycemic action. Furthermore, flavonoids have been shown to imitate the actions of insulin, enhance peripheral tissue glucose absorption, and it modifies the limiting enzymes' activity and/or rate of expression in metabolic pathways related to the metabolism of carbohydrates (Brahmachar 2011).

In conclusion, CTE increases the expression of the INS-1 gene and reduce TNF- $\alpha$  and IL-1 $\beta$  expressions. The conclusion, *C. ternatea* extract has potency as antidiabetic through increasing insulin level and inhibiting inflammatory activity.

## Conflict of Interest

No conflicts of interest were disclosed by the authors.

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