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Protein extracted from *Clitoria ternatea* modulates genes related to diabetes *in vivo*

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Abstract

Butterfly pea (*Clitoria ternatea*) is an indigenous plant from Moluccas in Indonesia. Due to its pharmacological properties, the flower of this plant is also used as a medicine in Ayurvedic practices in India. The present work was aimed to determine the antidiabetic activity of C. *ternatea* protein extract (PCT) via the inhibition of α-amylase activity *in vitro*, and the modulation of genes related to diabetes in diabetic mice *in vivo*. *C. ternatea* flower protein extract was prepared using isoprecipitation, and its efficacy as an antidiabetic candidate was tested *in vitro* through α-amylase inhibition assay, and *in vivo* through the measurement of blood glucose level and diabetes-related genes expression, including those that encode for peroxisome proliferator-activated receptors gamma (PPARγ), glucose transporter 2 (Glut2), transcription factor 7-like 2 (Tcf7l2), calpain-10 (Capn10), and monocyte chemoattractant protein 1 (MCP1) in alloxan-induced diabetic mice. PCT showed α-amylase inhibition in a dose-dependent manner. The highest α-amylase inhibition (20.63%) was observed at 1 mg/mL of PCT. The *in vivo* study showed that PCT decreased the blood glucose level in alloxan-induced diabetic mice. PCT also altered the expression of diabetes-related genes. In adipose and skeletal muscle tissues, PCT upregulated the gene expression of PPARγ and Tcf7l2, meanwhile MCP1 expression was downregulated. In the pancreas, PCT upregulated the gene expression of Glut2, Capn10, and Tcf7l2. These results indicate that PCT is a potential antidiabetic agent for the treatment of type 2 diabetes.

Introduction

Diabetes is becoming a major health problem as the prevalence of this disease is rapidly increasing worldwide. In 2015, diabetes affected an estimated of 415 million people worldwide, and 10 million cases of diabetes were from Indonesia (IDF Diabetes Atlas, 2015). Type 2 diabetes (T2D) is the most common disease among society, which accounts for approximately 90% of all diabetes cases (Singh, 2011). Generally, T2D develops in adults, but nowadays it has also been reported in children (Zimmet *et al.,* 2013). Therefore, the development of active compound, particularly those originating from natural products, is of importance to overcome this issue.

Butterfly pea (*Clitoria ternatea*) is originated from the Moluccas, Indonesia, where it is more commonly known as "*kembang telang*". This plant belongs to the Fabaceae family (Chu *et al.,* 2016). In traditional Ayurvedic medicine, this plant has been used as memory enhancing, anti-stress, anxiolytic, anti-depressant, anti-convulsant, tranquilising, and sedative agent (Chayaratanasin *et al.,* 2015). Almost all parts of this plant have been traditionally utilised to treat many diseases, such as urinary problems, eye swelling, night blindness, scabies, oedema, skin diseases, chronic bronchitis, goitre, leprosy, sore throat, and tumours (Desmukh and Jadhav, 2014; Mahmad *et al.,* 2016). The extract of *C. ternatea* posed several pharmacological effects such as antioxidant, hypolipidemic, anti-cancer, anti-inflammatory, analgesic, anti-pyretic, anti-microbial, and anti-diabetic activities (Al-Snafi, 2016).

Several researches have been conducted to investigate the anti-diabetic effects of *C. ternatea* extract. Methanol, water, petroleum ether, and chloroform extracts of *C. ternatea* leaves showed hypoglycaemic effects in streptozotocin-induced diabetic rats. The methanol extract of *C. ternatea* leaves also showed hypoglycaemic effect in alloxan-induced diabetic rats. Another hypoglycaemic effect was also shown by the aqueous extract of *C. ternatea* leaves and flowers in alloxan-induced diabetic rats (Al-Snafi, 2016). Our previous *in silico* and *in vivo* studies demonstrated that anthocyanin and ternatin extracted from *C. ternatea*

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flower exerted anti-inflammatory potency similar to that of ibuprofen standard (Wijaya *et al.,* 2020; Yanti *et al.,* 2020). The present work was conducted to determine the anti-diabetic activity of protein extract from *C. ternatea* flower (PCT) *in vitro* via α-amylase inhibitory assay, and *in vivo* via the measurement of blood glucose level and quantification of genes related to diabetes (PPARγ, Glut2, Tcf7l2, Capn10, and MCP1) in alloxan-induced diabetic mice.

Materials and methods

Extraction of protein from C. ternatea flowers

C. ternatea flowers were obtained from a local nursery in Bogor, West Java, Indonesia. Samples were sun-dried, and ground to a fine powder of approximately 60 mesh particle size. *C. ternatea* flower protein was extracted using isoelectric precipitation according to Salcedo-Chávez *et al.* (2002) with slight modification. Briefly, dried flower powder was dissolved in distilled water (10% w/v), followed by alkaline extraction at pH 8.6, adjusted with 1 M NaOH and stirring for 1 h at 40°C. The mixture was centrifuged at 10,000 *g* for 30 min at 25°C. To precipitate protein from the supernatant, the pH was adjusted to 4.5 using 2 M HCl. The suspension was incubated at 4°C overnight, followed by centrifugation at 10,000 *g* for 60 min at 25°C. The pellet was collected and stored at -20°C overnight prior to freeze-drying the following day. The dried protein extract of *C. ternatea* (PCT) was stored at -20°C for further study. The molecular weight of PCT was determined by SDS-PAGE according to Laemmli (1970).

α-amylase inhibitory assay

The α-amylase inhibition assay was performed according to Poovitha and Parani (2016) with slight modification. PCT was prepared in various concentrations (0.2 - 1.0 mg/mL). A total of 500 µL of PCT was mixed with 500 µL of 0.02 M sodium phosphate buffer (pH 6.9, adjusted with 0.006 M sodium chloride) containing 0.5 mg/mL of α-amylase (MP Biomedicals) and incubated at 25°C for 10 min. Following that, 500 µL of 1% (w/v) starch solution in 0.02 M sodium phosphate buffer (pH 6.9, adjusted with 0.006 M sodium chloride) was added to the mixture, and incubated at 25°C for 10 min. The reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid, followed by heating on a boiling water bath for 5 min. Samples were cooled down to room temperature, then diluted to 1:4 ratio with distilled water, and the absorbance at 540 nm was measured. Acarbose (Sigma) was used as standard. This assay was performed in triplicate. Anti-diabetic activity was represented by the percentage of α-amylase inhibition, which was calculated using Eq. 1:

$$\%\ Inhibition = \left(\frac{A_{540}control - A_{540}treatment}{A_{540}control}\right) x\ 100 \quad \text{(Eq.1)}$$

where, A_{540} control = absorbance at 540 nm with no PCT treatment, and A_{540} treatment = absorbance at 540 nm with PCT treatment.

Animal study and sample treatment

The animal models used for this experiment were 8 - 10 weeks old male ddY mice (25 - 30 g). These mice were kept in standard cages at room temperature (25 ± 1°C) under a 12 h light / 12 h dark cycle, and were fed with standard diet and water *ad libitum*. The PCT was administered in two dosages of 100 and 500 mg/kg body weight. Metformin standard was administered at a dose of 100 mg/kg body weight. All samples were dissolved in phosphate buffer saline. Diabetes induction was performed according to Szkudelski (2001) with slight modification. Mice were subjected to fasting for 15 h but had free access to drinking water. Diabetes was induced by intraperitoneal injection of alloxan at an increasing dose of 80, 100, and 150 mg/kg bw in 3 d interval. During the induction of diabetes, drinking water was replaced with 5% glucose. Ten days after the first alloxan injection, blood glucose levels were measured and mice with blood glucose level ≥ 200 mg/dl were considered as diabetic mice and selected for further study.

The experimental mice were divided into five groups containing five mice in each group (Table 1), including negative control (normal mice supplemented with phosphate buffer saline; NDC), positive control (alloxan-induced diabetic mice supplemented with phosphate buffer saline; DC), sample treatments (alloxan-induced diabetic mice supplemented with PCT at 100 and 500 mg/kg body weight; P100 and P500), and standard treatment (alloxan-induced diabetic mice supplemented with metformin at 100 mg/kg bodyweight; Met). Each group received single daily dose treatment through oral gavage for 30 d. Blood glucose levels were measured with commercially available diagnostic kits (Accu-Chek Active, Roche Diagnostic) at day 0, 15, and 30 after the first treatment. After 30 d, the animals were euthanised with 70 mg/kg bw ketamine. Animal experiments were conducted at VStem Animal Facility (Bogor, Indonesia) and the protocol was approved by the Animal Welfare Supervision Commission and Use of Research Animals. Adipose tissue, skeletal muscle tissue, and pancreas were collected and stored at -80°C for gene expression analysis.

Table 1. Treatment group of experimental mice.

Group	Treatment
NDC	Normal mice, phosphate buffer saline
DC	Alloxan-induced (AI), phosphate buffer saline
P100	AI + 100 mg/kg body weight PCT
P500	AI + 500 mg/kg body weight PCT
Met	AI + 100 mg/kg body weight metformin

Quantitative real time-PCR analysis

Tissue and organ samples in experimental mice, including adipose tissue, skeletal muscle tissue, and pancreas were used for gene expression analysis using qRT-PCR. Samples were disrupted using mechanical disruption by cryogenic grinding. The disruption was continued chemically using TRIzol (Life Technology, USA) following the manufacturer's protocol, and incubated at room temperature for 5 min. Chloroform was added for phase separation, incubated at room temperature for 2 - 3 min, and centrifuged at 12,000 *g* for 10 min at 4°C. The colourless aqueous phase on the top layer was transferred to new tube, and RNAs were precipitated using isopropanol. This mixture was incubated at room temperature for 10 min and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was removed and RNA pellet was washed with 80% ethanol. The final RNA pellet was dissolved in DEPC water, and RNA concentration was measured using NanoDrop. The expression of diabetes-related genes (PPARγ, GLUT2, Capn10, Tcf7l2, and MCP1) was measured by qRT-PCR. Primers were designed using the Primer-Blast software from National Center for Biotechnology Information (Table 2). cDNA synthesis was conducted using KAPA SYBR® FAST One-Step qRT-PCR Master Mix (Kapa Biosystems, USA). Thermocycling condition consisted of an initial polymerase activation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, and 55°C for 30 s. All gene expression values were normalised to β-actin-encoding gene as a reference. Expression levels were calculated by relative quantification using the $2^{-\Delta\Delta C}{}_{T}$ method, in which the data were log-transformed (log base of 2) and alterations with respect to the diabetic control group were displayed as log-fold changes (Maness, 2015).

Statistical analysis

All data were expressed as mean ± standard deviation (SD) of triplicates. Statistical analyses were performed with Statistical Package for Social Sciences (SPSS, USA). The significant difference between the means was determined using the Student's *t*-test. Statistical significance was accepted at $p < 0.05$.

Results

Molecular weight of PCT and effect of PCT on α-amylase inhibition

PCT was extracted using isoelectric precipitation and characterised by SDS-PAGE. PCT consisted of two protein bands with molecular weights of 60 and 80 kDa (Figure 1a). The inhibition of α-amylase activity by PCT and acarbose standard was dose-dependent, as shown by an increment of 0.2 to 1.0 mg/mL (Figure 1b). The highest α-amylase inhibition was observed at 1.0 mg/mL of PCT (20.63%), while at the same concentration, acarbose showed 14.30% inhibition of α-amylase.

Effect of PCT on blood glucose level in diabetic mice

Administration of alloxan to normal mice generally resulted in the increase of blood glucose level as compared to the control group. PCT and metformin treatment in alloxan-induced diabetic mice resulted in a decrease of blood glucose level,

Table 2. Primers used for qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product length (bp)
PPARγ	CATCAGGTTTGGGCG GGAT	TGTGATCTCTTGCACGGCTT	355
GLUT2	AAGCCACAAGTCATTGGGGT	GGTAGCCTGACCTGTGGTAAC	329
Capn10	TTCCAGCAGACGGTGAGAAC	GGTCTCGTGTCAGGCTTTCA	284
Tcf7l2	CGCACTTACCAGCTGACGTA	GTCACAGCGACTCACCTAGA	226
MCP-1	TCCCAAGAGGTACCGCAAAC	TACCAGGAGCCAGGCATAGT	322
β-actin	AGGGAAATCGTGCGTGACAT	ACTGTGTTGGCATAGAGGTC	278

which relatively sustained throughout 30 d of observation (Figure 2). The maximum reduction in blood glucose level was achieved through the administration of 500 mg/kg bw PCT. This effect was comparable to metformin at dosage of 100 mg/kg bw.

Effect of PCT on diabetes-related genes expression

The effect of PCT and metformin on the expression of diabetes-related genes, including PPARγ, GLUT2, Capn10, Tcf7l2, and MCP1 in alloxan-induced diabetic mice was measured by qRT-PCR (Figure 3). In adipose and skeletal muscle tissues, PPARγ and Tcf7l2 were upregulated, while MCP1 was downregulated. In the pancreas, Glut2, Capn10, and Tcf7l2 were upregulated. PPARγ in adipose tissue was significantly higher ($p < 0.05$) in the PCT (100 and 500 mg/kg bw) and metformin (100 mg/kg bw) treated groups as compared to in the diabetic mice group. Tcf7l2 was also upregulated by PCT and metformin in the adipose tissue, but there was no significant difference as compared to the diabetic mice group. On the other hand, MCP1 was significantly downregulated by PCT (500 mg/kg bw) and metformin (100 mg/kg bw) ($p < 0.05$) as compared to the diabetic mice group (Figure 3a).

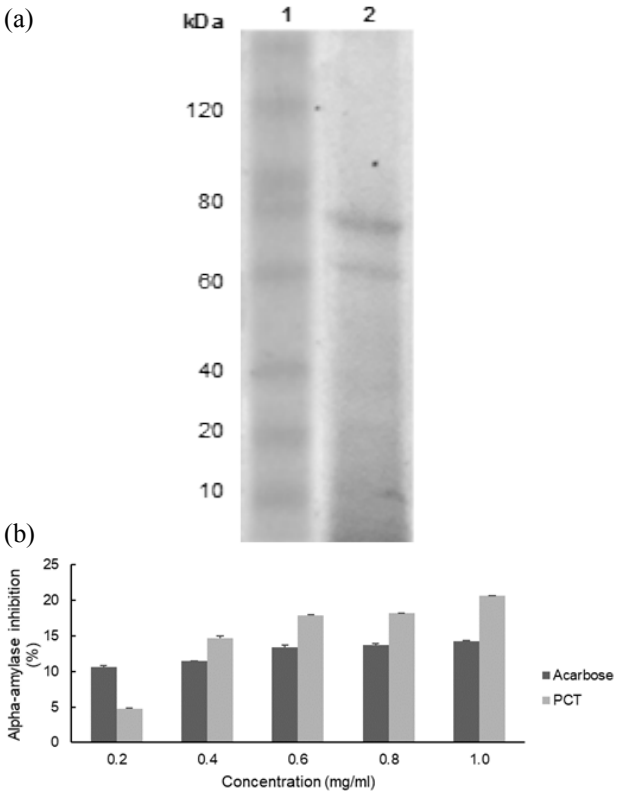

Figure 1. Molecular weight of protein from *C. ternatea* (PCT) by SDS-PAGE assay (a), and its efficacy on inhibiting α-amylase activity *in vitro* (b). Lane 1: protein marker; and Lane 2: *C. ternatea* protein. Acarbose was used as reference standard. Values are means ± SD.

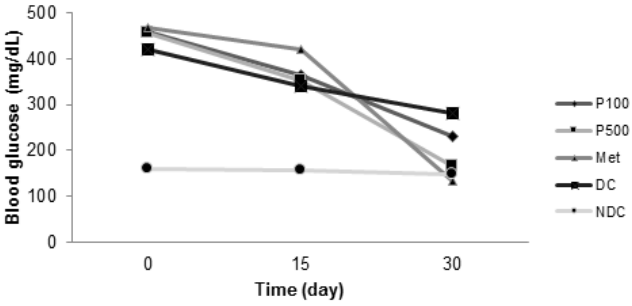

Figure 2. Blood glucose level of five groups of male ddY mice at day 0, 15, and 30 after the first oral administration of samples. P100: diabetic mice + *C. ternatea* protein (100 mg/kg bw); P500: diabetic mice + *C. ternatea* protein (500 mg/kg bw); Met: diabetic mice + metformin (100 mg/kg bw); DC: diabetic control (phosphate buffer saline); and NDC: non-diabetic control (phosphate buffer saline).

In the skeletal muscle tissue, PPARγ was upregulated by PCT and metformin, but only metformin (100 mg/kg bw) treatment resulted in a significant increase ($p < 0.05$) as compared to the diabetic control group. PCT (500 mg/kg bw) and metformin (100 mg/kg bw) treatment led to the upregulation of Tcf7l2 expression ($p < 0.05$). Meanwhile, PCT (100 and 500 mg/kg bw) and metformin (100 mg/kg bw) supplementation resulted in the downregulation of MCP1 ($p < 0.05$) in skeletal muscle tissue relative to the diabetic control group (Figure 3b).

In the pancreas, the Glut2, Capn10, and Tcf7l2 genes were upregulated (Figure 3c). Significant upregulation of Glut2 ($p < 0.05$) was shown for PCT treatment (500 mg/kg bw), while there was no significant difference of Capn10 expression in mice groups treated with either PCT or metformin. Tcf7l2 was significantly upregulated ($p < 0.05$) by PCT (100 and 500 mg/kg bw).

Discussion

C. ternatea has been widely used in traditional Ayurvedic medicine, and several studies have been conducted to elucidate the pharmacological activity of this plant. The present work was focused on elucidating the anti-diabetic activities of *C. ternatea* extract. Current therapeutic strategy for diabetes, in particular to control the postprandial hyperglycaemia, involves the inhibition of carbohydrate-digesting enzyme, resulting in aggressive delay of carbohydrate digestion to absorbable monosaccharide (Raptis and Dimitriadis, 2001). α-amylase is an example of carbohydrate-digesting enzyme. Adisakwattana *et al.* (2012) have reported the *in vitro* inhibitory effect of *C. ternatea* extract on pancreatic α-amylase, yet no report is available for α-amylase inhibitory activity of protein extract derived from this plant. The present

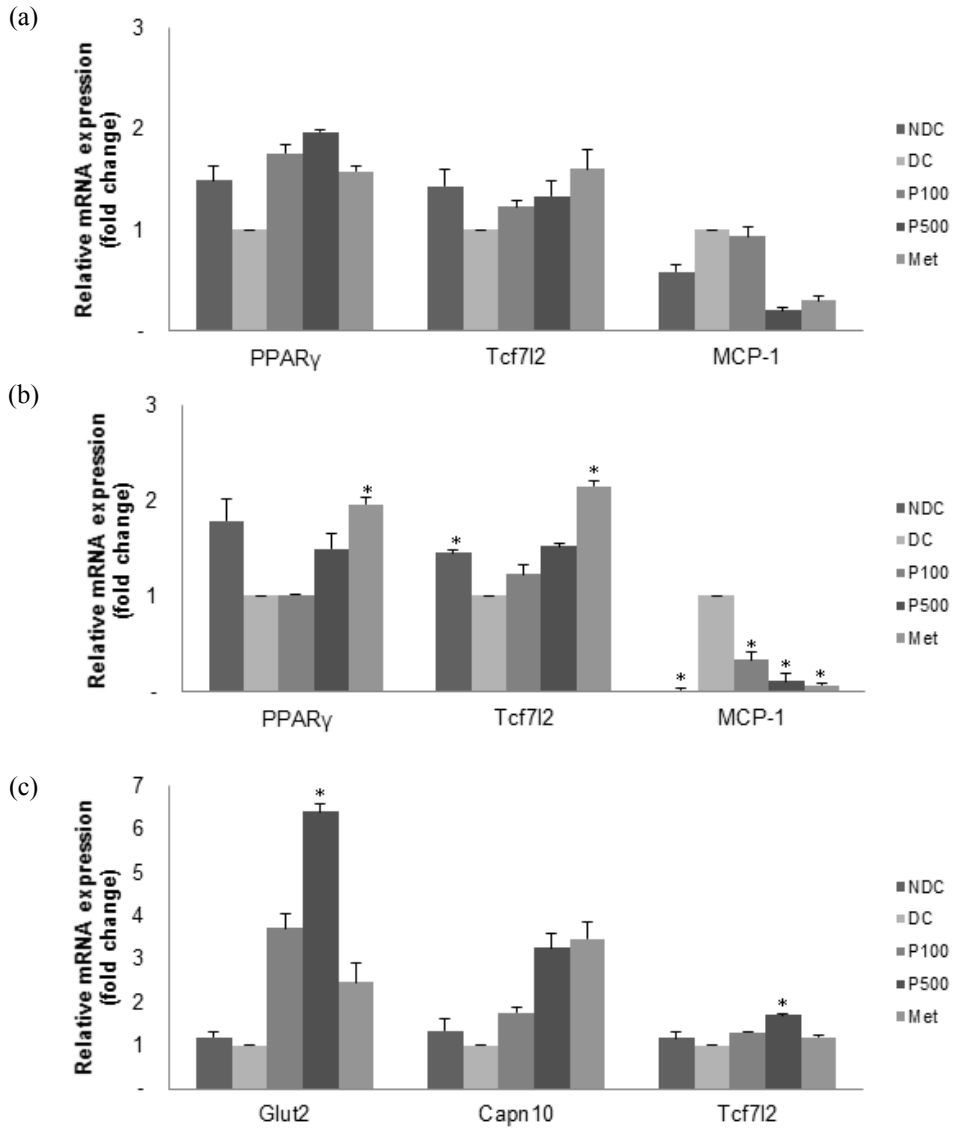

Figure 3. Relative expression of diabetes-related genes in adipose tissue (a), skeletal muscle tissue (b), and pancreas (c) of five groups of male ddY mice: non-diabetic mice (NDC), diabetic mice (DC), *C. ternatea* protein at 100 mg/kg bw (P100), *C. ternatea* protein at 500 mg/kg bw (P500), and metformin at 100 mg/kg bw (Met). Values were normalised to β-actin mRNA levels and were expressed relative to diabetic control group (DC). Values are means ± SD. * = $p < 0.05$ as compared to the DC group.

work confirmed that PCT containing two major proteins (60 and 80 kDa) also exerted α-amylase inhibition (Figure 1). α-amylase is one of the key enzymes in carbohydrate metabolism (Liu *et al.,* 2011). This enzyme cleaves the α-1,4-glucosidic bonds in starch and to hydrolyse it into smaller oligosaccharides, which are then hydrolysed by α-glucosidase to glucose (Gomathi *et al.,* 2012). Inhibition of this enzyme can contribute to the management of T2D by reducing the rate of glucose absorption (Ademiluyi and Oboh, 2012). The α-amylase inhibitory property of PCT can be applied in controlling postprandial hyperglycaemia in T2D by retarding glucose absorption through the inhibition of carbohydrate-hydrolysing enzyme, i.e. α-amylase. Thus, carbohydrate cannot be hydrolysed to oligosaccharide and resulted in less oligosaccharide to be hydrolysed to glucose.

` The intraperitoneal administration of alloxan can induce diabetes via the establishment of redox cycle with the formation of superoxide radicals, which then undergo dismutation resulting in hydrogen peroxide. Thereafter, highly reactive hydroxyl radicals are formed through Fenton reaction. The action of reactive oxygen species (ROS) with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β-cells (Szkudelski, 2001).

Diabetes in mice, which was induced by alloxan, is indicated by the elevated blood glucose

levels. Alloxan-induced mice showed higher blood glucose level as compared to the normal mice group. Diabetic mice treated with PCT and metformin showed reductions in the blood glucose level as compared to diabetic mice group (Figure 2). Metformin decreased hepatic glucose production through the activation of 5'adenosine monophosphate-activated protein kinase (AMPK) pathway (Foretz *et al.,* 2010). Meanwhile, the therapeutic mechanism of PCT in reducing blood glucose level is still unknown and further study is required to further elucidate this

PCT modulated the expression of diabetes-related genes in diabetic mice (Figure 3). The expressions of PPARγ, Tcf7l2, Glut2, and Capn10 genes were upregulated by PCT, while MCP1 was downregulated. The increased expression of PPARγ, Tcf7l2, Glut2, and Capn10 indicate that PCT plays a role in enhancing insulin sensitivity and insulin secretion. The downregulated expression of MCP1 indicates that PCT plays a role in reducing inflammation related to diabetes. PPARγ has a critical role in the pathogenesis of insulin resistance because this gene controls a set of genes that are involved in glucose metabolism (Ruschke *et al.,* 2010). At cellular level, PPARγ activation has been shown to affect the insulin signalling cascade through direct modulatory effects on the expression and/or phosphorylation of specific signalling molecules (Leonardini *et al.,* 2009). In addition, Wu *et al.* (1998) also reported that PPARγ activation directly regulated the expression of glucose transporter-encoding GLUT4, which was the key modulator of glucose disposal in adipose and muscle tissues. The upregulated expression of PPAR γ by PCT showed that this protein stimulates the activation of this gene (Figure 3a). The stimulation of PPARγ may enhance insulin sensitivity in adipose and skeletal muscle tissues by promoting glucose uptake via GLUT4. Furthermore, bloodstream glucose can enter the target tissues, i.e. the adipose and skeletal muscle tissues, and maintain normal level of glucose in the blood. These results are in accordance with the blood glucose level data in our experiment (Figure 2 and 3).

MCP1 has been reported to play a role in the development of insulin resistance. The protein is a marker of inflammation that inhibited insulin signal transduction and led to insulin resistance in adipose and skeletal muscle tissues (De Luca and Olefsky, 2008). The inflammation in insulin-target tissues, such as adipose and skeletal muscle, could be localised or systemic. Localised insulin resistance is caused by chronic inflammation via autocrine/paracrine signalling, where the systemic insulin resistance occurs via endocrine cytokine signalling (Panee, 2012). Downregulation of MCP1 in adipose and skeletal muscle tissues by PCT showed that the product of this gene may suppress inflammation related to diabetes and inhibit the development of insulin resistance in insulin-target tissues.

Tcf7l2 is a member of the high mobility group box family of transcription factors, activated by the Wnt signalling pathway (Pang *et al.,* 2013). The signalling protein insulin receptor substrate-1 (IRS-1) is a transcriptional target of Wnt signals. Increased level of IRS-1 in insulin-responsive cell types enhanced insulin signalling, indicating that Wnt proteins play a role in glucose homeostasis (Yoon *et al.,* 2010). In the adipose and skeletal muscle tissues, the upregulation of Tcf7l2 by PCT showed that this protein might play a role on the activation of Wnt signalling pathway, which further increased the level of IRS-1 in adipose and skeletal muscle tissues. This process would enhance insulin signalling. As the adipose and skeletal muscle tissues are more sensitive to insulin, this would allow for glucose disposal in these tissues and maintain normal blood glucose. These results are in accordance with the blood glucose level data in our experiment (Figure 2 and 3).

Tcf7l2 was also involved in stimulating the proliferation of pancreatic β-cells (Jin and Liu, 2008). The depletion of Tcf7l2 led to a decrease in β-cells proliferation and glucose-stimulated insulin secretion. The depletion of this transcription factor also resulted in the increasing number of β-cells apoptosis. In the pancreas, the upregulation of Tcf7l2 by PCT showed that the product of this gene may play a role in the survival of pancreatic β-cells and glucose-stimulated insulin secretion. Further study is required to confirm the correlation among PCT, Tcf7l2, and insulin secretion.

GLUT2 is glucose transporter in mammalian cells encoded by Glut2. GLUT2 is mainly responsible for glucose entry in pancreatic β-cells and required for glucose-stimulated insulin secretion (Thorens, 2015). Previous study demonstrated that Glut2 was under expressed in pancreatic islets of numerous animal models of non-insulin-dependent diabetes mellitus. Such molecular defect has been proposed to contribute to the pathogenesis of diabetes. In the pancreas, the upregulation of GLUT2-encoding gene by PCT showed that this protein played a role in glucose metabolism by stimulating glucose uptake to the pancreas and initiating cellular insulin secretion via exocytosis, followed by binding to specific receptors in the insulin-target tissues and maintaining glucose homeostasis. Consistently, these results are in accordance with the blood glucose level data in our experiment (Figure 2 and 3).

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Capn10 is a calcium-dependent intracellular non lysosomal protease that can hydrolyse important substrates in calcium-regulated signalling pathway. Capn10 has been suggested to influence insulin secretion. In insulin secretion mechanism, the increasing level of calcium in β-cells triggers the cells to secrete insulin via exocytosis (Marshall *et al.,* 2005). In the present work, the upregulation of Capn10-encoding gene by PCT showed that this protein might modulate insulin secretion by triggering the exocytosis of insulin granules in pancreatic β-cells. However, further study is required to determine the association among PCT, Capn10, and insulin secretion.

Conclusion

Protein extract from *C. ternatea* flower (PCT) effectively inhibited α-amylase *in vitro* and lowered blood glucose level *in vivo* in alloxan-induced diabetic mice. This protein also significantly altered the expression of several genes related to diabetes, including PPARγ, Tcf7l2, Glut2, Capn10, and MCP1 in alloxan-induced diabetic mice. Therefore, *C. ternatea* protein may play role in management of type 2 diabetes via the modulation of gene expression related to diabetes and inhibition of α-amylase activity.

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