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Research Article

Extract of Torbangun (*Coleus amboinicus* Lour) Leaves Inhibits the Expression of *PEPCK* and *G6Pase* in Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Objective: This study aimed to analyze the anti-hyperglycemic activity of Torbangun leaf extract (TLE) in hyperglycemic rats by observing its effect on blood glucose levels and *PEPCK* and *G6Pase* gene expression. **Method:** This post-test-controlled group design study used a completely randomized design (CRD). Seven rats were separated as a normal group (N) and other rats were injected with streptozotocin (STZ). Hyperglycemia was characterized by fasting blood glucose (FBG) >126 mg/dl. Hyperglycemic rats were divided into three groups: H-IM (control drug metformin hydrochloride dose 62.5 mg/kg BW), H-IT (TLE dose 620mg/kg BB), and NG (negative control, hyperglycemic rats). Rats were given intervention for 14 days. **Result:** The results showed a significant difference between initial and final FBG of the HI-IM group ($p = 0.002$, $\alpha = 0.01$) and the H-IT group ($p = 0.005$, $\alpha = 0.01$) and effect on the key gene expression of gluconeogenesis in the liver. The H-IT group showed a decrease in PEPCK gene expression 0.80 times and decreased G6Pase gene expression by 0.65 times compared to NG group. **Conclusion:** The findings suggested the administration of TLE in STZ-induced hyperglycemic rats was able to reduce blood sugar levels and was involved in inhibiting the expression of PEPCK and G6Pase expression genes in the liver.
Key words: G6Pase, Hyperglycemia, PEPCK, Rats, Torbangun.

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INTRODUCTION

The liver plays an important role in blood glucose homeostasis by maintaining a balance between glucose absorption and storage through glycogenesis and glucose release through glycogenolysis and gluconeogenesis. The level of gluconeogenesis is controlled primarily by the activity of several key enzymes, including phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase).[1]

PEPCK is an enzyme that controls the rate of hepatic gluconeogenesis and plays an important role as a centre for regulating glucose hemostasis. PEPCK is the first step in gluconeogenesis in the liver.[1,2] Besides PEPCK, there are also G6Pase enzymes in the liver which are found in the endoplasmic reticulum. This enzyme plays a role in the formation of free glucose from glucose-6-phosphate (G6P) in the final step of gluconeogenesis and glycogenolysis.[3]

The rate of transcription of the *PEPCK* gene is enhanced by glucagon (*via* cAMP) and glucocorticoids and inhibited by insulin. In addition, under certain conditions, glucose is also able to increase the expression of *PEPCK* genes.[4,5] Supplement that usually *PEPCK* expression is induced by glucagon, catecholamines and glucocorticoids during the fasting period and response to stress, but are predominantly inhibited by increases in glucose induced insulin secretion in food.[2] Meanwhile, the catalytic subunit of *G6Pase* is coded by one of three genes, namely *G6PC1*, *G6PC2* and *G6PC3*.[6] At certain conditions, the expression of the *G6PC1* gene is directly regulated by insulin and glucagon.[7]

Chronic hyperglycemia will affect the gene expression of regulating hepatic glucose output and gluconeogenesis. This is explained by Shao *et al* (2005) that sustained hyperglycemia can reduce the effects of glucose and insulin inhibition on *PEPCK* gene expression and increase the expression of the *PEPCK* gene hormone stimulant, as well as liver glucose production.[1] Furthermore, *G6Pase* gene expression is enhanced by high glucose concentrations. This shows that hyperglycemia can increase G6Pase activity.[8]

Continuous hyperglycemia causes an increase in free radical production called ROS (Reactive Oxygen Species). Increased ROS is associated with the development of diabetes mellitus (DM) because it can cause a decrease in enzymatic antioxidant activity, causing tissue susceptibility to oxidative stress.[9]

One way to control DM is through nutraceutical consumption or functional food. There have been more than 1,050 anti-DM plants that have been studied.[10] Torbangun leaves are traditionally used to stimulate the production of breast milk from lactagogum as the local wisdom of the Batak people in North Sumatra.[11-14] Besides being used as lactagogum, Torbangun leaves are then used widely as antibacterial and antifungal,[15] blood pressure and cholesterol control.[16-18]

Several studies have reported the ability of Torbangun leaves has been used in DM therapy. A study conducted by Viswanathaswamy *et al* reported that ethanol extract of Torbangun leaves improved pancreatic function and insulinotropic effect.[18]

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Ethanol extract of Torbangun leaves contains flavonoids as bioactive compounds at a dose of 620 mg/kg BW for 14 consecutive days significantly reduced blood glucose and free radical levels, increased the glucokinase enzyme, and protected cells pancreatic-β cells from STZ-induced rats.[16] One type of flavonoids, namely quercetin, was able to stimulate glucose uptake in an insulin-dependent mechanism involving AMPK (protein kinase activated by adenosine monophosphate). In the liver, AMPK reduces glucose production through the reduction of key enzymes of gluconeogenesis, PEPCK, and G6Pase.[19]

So far, there is no information about the effect of Torbangun leaves extract (TLE) on glucose homeostasis key genes expression in the liver, namely *PEPCK* and *GP6ase*. Therefore, it is necessary to do a study to explain the mechanism of TLE in influencing the expression of these genes. The results of this study will make a fundamental contribution to find molecular theory at the genetic level about the mechanism of TLE as a potential anti-DM agent and will provide stronger scientific evidence about the application of complementary therapies for DM patients. Therefore, the purpose of this study is to analyze the anti-hyperglycemic activity of TLE in hyperglycemic rats by observing the effect of TLE on blood glucose levels and *PEPCK* and *G6Pase* genes expression.

METHODS

Main materials

Torbangun samples have been authenticated by the Botanical Gardens Conservation Center, Indonesian Institute of Sciences, Bogor, West Java, Indonesia (No B-2096/IPH.3/KS/VII/2017. The Torbangun seeds were planted in a total area of ±160 m^2 with a spacing distance of 40 cm[20] in Cibeureum, Bogor City, West Java, Indonesia, at an altitude of 298 asl. Torbangun leaves are harvested at the age of 8 weeks, taken about 15 cm from the top of the plant.[21]

The experimental animals used were 25 male Sprague-Dawley rats aged 8 weeks, with the minimum body weight of 140 g (148-192 g) from PT. Indoanilab Bogor, Indonesia. Standard rat feed was obtained from PT Indonesia Formula Feed.

STZ from Sigma-Aldrich has been used a single dose of 40mg/kg BW through intraperitonial.[22] DM drugs for the control group are metformin hydrochloride dose 62.5 mg/kg BW[23] from Dexa Medica, Indonesia. The anesthetics used are ketamine and xylazine.[24] Other laboratory materials are 96% ethanol, insulin level analysis kits from the Bioassay Technology Laboratory, China Qiagen Rneasy Insulation Mini Kit, and ReverTra Ace qPCR RT Master Mix with gDNA Toyobo Remover.

Extraction

Fresh Torbangun leaves was washed, dried at 40°C for three days using a fresh dryer and mashed with a grinder then sifted to a size of 60 mesh.[16] Torbangun leaves powder was extracted following the steps of Suryowati which were modified by adding a sonicator tool.[16]

The extract was made by mixing 25 grams of simplicia powder with Torbangun leaves as much as 25 grams into 250 ml ethanol 96% and then sonicated with Powersonic sonicator 505, 40 kHz for 40 minutes at room temperature (25°C). The extract was filtered with Whatman filter paper.[25] Sonication was repeated twice with the addition of 100 ml ethanol each repetition. Solvent evaporation in extracts was carried out with a rotary evaporator temperature of 60°C until semi-solid liquid was achieved.[18] The extract obtained was stored in an air tight container.[16]

Animal intervention

Twenty-five rats were kept in cages, separated individually by standard feeding. Seven rats were separated as normal (N) and the remaining rats were injected with STZ with a single dose of 40 mg/kg BW.[22] Verification of the hyperglycemia is characterized by a fasting blood glucose (FBG) above 126 mg/dL.[26, 27] All rats that received STZ induction experienced hyperglycemic on the third day after injection. Rats confirmed hyperglycemia were divided into groups: NG (control, hyperglycemic rats, 6 rats), H-IM (control of metformin drugs 62.5 mg/kg BW, 6 rats), and H-IT (TLE 620 mg/kg BW, 6 rats). The dose of Torbangun leaves extract is based on Suryowati *et al.*[16] The administration of TLE and metformin were carried out with the sonde on the same schedule every day for 14 days. TLE was dissolved with NaCMC 0.3% w/v and metformin was dissolved with distilled water to facilitate administration through a sonde.[16] All treatments received a standard ration containing 40% protein, 4% fat, 5% crude fiber, 8% ash, 1% Ca mineral, 0.8% mineral P, with 12% moisture content. FBG was taken on days 0, 4^{th}, 7^{th}, 11^{th} and 14^{th}, measured using glucometers. The rat's blood is taken from the tip of their tail and dropped on a glucometer strip.

At day 15 after the intervention, all rats were treated with a mixture of ketamine: 90 mg and xylazine: 10 mg dissolved in distilled water. Necropsy is done by cutting the skin and abdominal muscles until the abdominal cavity opened. The soleus muscle is stored in later RNA and stored at -20°C until the sample is ready for analysis.

Gene expression analysis

Liver was collected for gene expression analysis and stored in later Ribonucleid acid (RNA) liquids at -20°C until samples was ready for analysis. The expression of the gene was carried out by the reverse transcriptase polymerase chain reaction (RT-PCR) technique. The liver was frozen in liquid nitrogen and then stored at -70°C for the determination of the transcription gene. Qiagen Rneasy Mini Isolation Kit was used for RNA purification and Ace qPCR Rever-Tra RT Master Mix with Toyobo's gDNA Remover for reverse transcriptase. The tool used is Analytic Jena's q-TOWER with Thunderbird qPCR Green Sybr Master Mix Toyobo. The housekeeping gene used was *18sRNA*.[28-30] Primers design used National Center for Biotechnology Information (NCBI) Pubmed software blast primer. RT-PCR was carried out under the following conditions: 40 cycles for 1 minute at 95°C for pre-denaturation, 15 seconds at 95°C for denaturation, and 1 minute at 60°C for the annealing process. The primers of each gene are presented in Table 1.

Gene expression was calculated based on the approach of the relative number of mRNAs from the target gene with gene control (*18SRNA*) using the Comparative Ct method (ΔCt), expression between target genes and control genes can be compared with 2-ΔCt equations, with ΔCt = Ct-gen Ct control target gene (*housekeeping genes*).[31]

Statistical analysis

Data was recorded and initial processing was performed using MSExcel software. Differences in blood glucose levels before and after the intervention were analyzed by Paired T-Test Samples. Differences in insulin levels were analyzed by variance (ANOVA) at a 99% confidence interval to see whether there was an effect of treatment, and gene expression was analyzed descriptively.

Ethical considerations

The research protocol implemented has been approved by Ethical Treatment No. 77-2017 from the Animal Ethics Commission, Institute for Research and Community Service, IPB University, Indonesia.

RESULT

Table 2 gives the effect of giving TLE to the blood levels of hyperglycemic rats. A higher FBG level in the STZ-induced group at

Table 1: Primer gen target dan *housekeeping gene*.

Primers	Sequence 5'-3'	Access gene	Length
18sRNA F	CTT CTT AGA GGG ACA AGT GG	NR_046237.1	164 bp
18sRNA R	CAT CAC GAA TGG GGT TCA AC		
PEPCK (GTP) F	GTG ATG ACA TTG CCT GGA TG	NM_198780.3	126 bp
PEPCK (GTP) R	GAT GGT CTT AAT GGC GGT CG		
G6Pase F	GAA TGT CGT CTT GTG GTT GG	NM_013098.2	196 bp
G6Pase R	CAG GAA GAA GGT GAT GAG AC		

Table 2: Rats' FBG levels before and after the intervention.

No	Groups	Initial FBG (mg/dl)	Final FBG (mg/dl)	p
1	NG	153.000±3.559	167.330±14.582	0.350
2	N	100.86±6.197	91.290±8.237	0.462
3	H-IM**	147.83±6.838	103.170±2.212	0.002**
4	H-IT**	155.83±9.555	105.170±5.043	0.005**

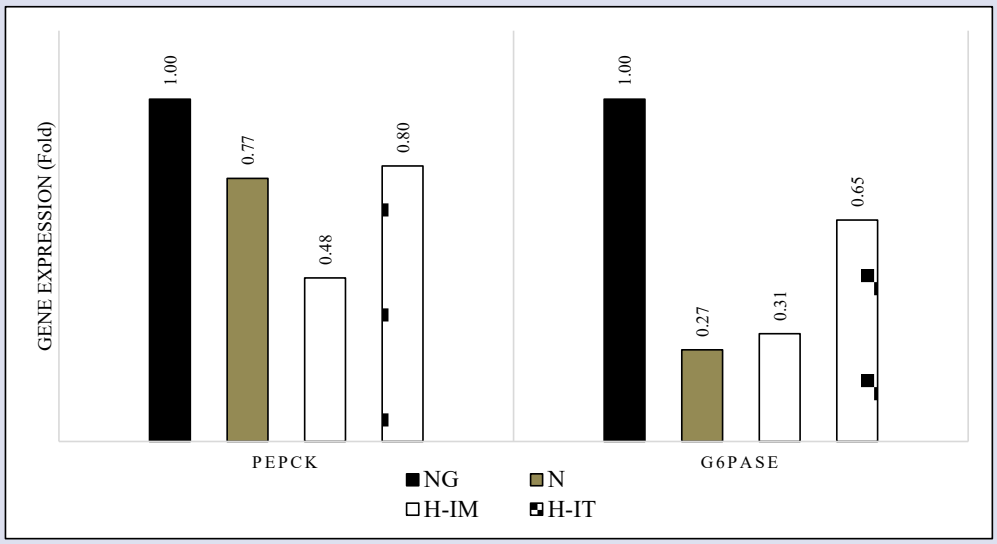

Figure 1: *PEPCK* and *G6Pase* gene expression at transcription level

NG=hyperglycemic rats, N=normal, H-IM=metformin drug control, H-IT=TLE

the start of the comparison showed that the object of the study was increased hyperglycemia as one of the symptoms of DM. The FBG level of the group of rats that were intervened with TLE and the metformin drug control group showed a decline towards normal conditions at the end of the study. Meanwhile, the NG group still hyperglycemia, FBG > 126 mg/dl). The results of the statistical analysis revealed a significant difference between initial and final FBG on the HI-IM group ($p = 0.002$, $\alpha = 0.01$) and the H-IT group ($p = 0.005$, $\alpha = 0.01$).

This study shows that the administration of TLE in hyperglycemic rats has an effect on the key gene expression of gluconeogenesis in the liver (*PEPCK* and *G6Pase*). The group of rats given the TLE intervention (H-IT) showed a decrease in *PEPCK* gene expression 0.80 times compared to the group of hyperglycemic rats (NG).

TLE also decreased *G6Pase* gene expression by 0.65 times. Although the decrease in value was not as large as the N and H-IM groups, it was seen that the intervention of TLE was able to reduce the expression of *PEPCK* and *G6Pase* genes, thereby reducing the rate of gluconeogenesis and reducing glucose levels in the blood (Figure 1).

DISCUSSION

The pathogenesis of DM is characterized by circulation, namely a decrease in peripheral tissue response to insulin.[32] Damage to tissues occurs due to an increase in free radicals in the body. Also, the ability of insulin to increase compatibility.[33]

The results of the statistical analysis revealed a significant difference between the initial and final FBG interventions in the H-IT and H-IM groups. This shows the effect of Torbangun's extract in reducing blood levels in hyperglycemic rats, having the same complexity as metformin. According to,[18] a decrease in blood glucose levels in rats given TLE was carried out through the restoration of pancreatic tissue function and insulinotropic effects. This happens because the antioxidants contained in TLE can trigger free radicals that are formed due to hyperglycemia. Decreased levels of free radicals cause insulin receptors to work properly, thus, carrying can be transported into cells, producing glycogenesis and reducing the rate of gluconeogenesis.

TLE contains flavonoids with potential antioxidant effects.[34] The results of this study are consistent with Viswanathaswamy *et al* who reported that TLE can improve the function of pancreatic tissue and insulinotropic effects (production, secretion, and insulin activity).[18]

The liver plays a major role in blood glucose homeostasis by maintaining a balance between glucose uptake and storage through glycogenesis and glucose release through glycogenolysis and gluconeogenesis. Glucose homeostasis in the body is highly dependent on genes that regulate the formation of enzymes that play a role in glycogenolysis and gluconeogenesis.[35]

The key liver genes that govern the process are *PEPCK* and *G6Pase*.[36] The bioactive component in plants can influence insulin signaling, which can change the expression of the key gene for glucose homeostasis.[37]

Chronic hyperglycemia leading to DM reduces the ability of glucose and insulin to inhibit *PEPCK* gene expression. This increase in *PEPCK* gene expression increases the rate of gluconeogenesis, resulting in increased liver glucose production.[1] There were many known pharmaceutical preparations and plant extracts that had anti-hyperglycemia ability and inhibited *PEPCK* gene transcription.[38-45] Metformin plays a role in increasing the secretion of endorphins from the adrenal gland to stimulate receptor-opioid relationships, causing down-regulating of *PEPCK* gene expression in the liver.[23]

The process of decreasing *PEPCK* and *G6Pase* gene expression in the liver in the H-IT group can be explained by the following two mechanisms. *First*, it is related to the improvement of pancreatic-β cells, so that the production and effectiveness of insulin improves. The pancreas of DM rats that were treated with the extract of the leaves of Torbangun experienced regeneration changes to normal. Giving TLE can protect endocrine cells due to STZ induction so that insulin secretion can be increased.[16] *PEPCK* expression up-regulation is influenced by insulin.[39] Insulin and glucagon regulate protein expression and activity to maintain blood glucose levels. The main target is PEPCK, an enzyme that is the first catalyst in the process of gluconeogenesis. The regulation of the *PEPCK* gene is achieved by modulating the transcription of tightly regulated genes by cAMP (glucagon and catecholamine mediators), glucocorticoids, and insulin.[2] *Second*, there is a stimulation of the flavonoids contained in TLE against AMPK (adenosine monophosphate-activated protein kinase). As stated by Eid *et al* that AMPK decreases glucose production primarily through the regulation of key enzymes of gluconeogenesis.[19] Suryowati suspects that the antioxidant quercetin contained in the leaves extract of Torbangun is one of the antioxidants that play a role in stimulating glucose uptake involving AMPK.[16]

CONCLUSION

The findings suggested the administration of TLE in STZ-induced hyperglycemic rats was able to reduce blood sugar levels and was involved in inhibiting the expression of key genes of gluconeogenesis (*PEPCK* and *G6Pase*) in the liver.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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