

The Effect of Puguntano Leaf Extract (*Curanga Fel - Terrae Merr.*) On P38 Mapk Levels and Glut-4 Expression in Type 2 Diabetic Rat Muscle

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Abstract

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BACKGROUND: Puguntano (*Curanga feel-terrae Merr.*) contains flavonoids, saponins, tannins, and steroids/terpenoids which improved post-receptor insulin signalling in rats model of type 2 diabetes mellitus (T2DM).

AIM: This study aimed to determine the effect of puguntano leaf extract on p38 mitogen-activated protein kinase (MAPK) levels and glucose transporter-4 (GLUT-4) expression in diabetic rats muscle.

METHODS: Forty-eight male Wistar rats had T2DM induced using a combination of feeding a high-fat diet for 5 weeks and multiple intraperitoneal injections of low-dose streptozotocin (30 mg/kg). The diabetic rats were randomly divided into control and treatment groups, and 200 mg/kg/day puguntano extract was administered orally for 10 days to treatment group. Subsequently, p38 MAPK levels were measured by Sandwich Elisa and plasma membrane GLUT-4 expression was evaluated by Immunohistochemistry in their gastrocnemius muscles.

RESULTS: There were significantly higher p38 MAPK levels and GLUT-4 expression in the treatment group than in the control group.

CONCLUSION: These data suggest that a puguntano leaf extract can improve post-receptor insulin signalling by enhancing p38 MAPK levels and GLUT-4 expression in a rat model of T2DM.

Introduction

Diabetes mellitus (DM) is a chronic condition characterised by high blood glucose concentration resulting from insufficient insulin production and/or ineffective insulin action [1]. The International Diabetes Federation (IDF) reported in 2017 that there were 10.3 million people with diabetes in Indonesia,

and this number was estimated to increase to 16.7 million by 2045. Indonesia is in the 6th rank of the top ten countries for some people with diabetes of all the countries of the world [2].

Insulin is an anabolic hormone secreted by pancreatic β -cells that regulate a wide range of physiological processes. Normal metabolism requires the coordinated secretion and action of insulin, but in type 2 diabetes (T2DM), both its action and secretion

are impaired [3].

Insulin resistance in the insulin-sensitive tissues, such as liver, muscle, and fat, is the principal feature of T2DM [4]. Skeletal muscles play an important role in insulin-mediated glucose uptake in the post-prandial state (80%) [5]. In the initiation stage of the insulin signalling pathway, binding of the insulin to its receptor is followed by intracellular signalling via two main downstream pathways, i.e. mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol-3 kinase (PI3K) pathway [6]. Activation of PI3K pathways promotes glucose uptake by increasing the translocation of glucose transporter-4 (GLUT-4) from intracellular vesicles to the plasma membrane of skeletal muscle, facilitating glucose uptake [5]. p38 MAPK has also been shown to be necessary for regulation of insulin-stimulated glucose uptake through GLUT-4 in response to insulin, but findings regarding the potential role of p38 MAPK in the regulation of glucose transport in skeletal muscle remain controversial [7]. GLUT-4 plays an important role in the maintenance of glucose homeostasis via its translocation and expression in skeletal muscle [5]. A great deal of evidence implicates defects in post-receptor signalling as the major cause of insulin resistance in target tissues [8], including defects in GLUT-4 expression and function [9].

Puguntano (*Curangafel-terrae* Merr), a medicinal plant from Scrophulariaceae family, grows in Asia especially in China, India, Indonesia, Philippines, Malaysia and Myanmar. Puguntano leaves from the Dairi area of North Sumatera Province have long been used empirically to control blood glucose levels. Puguntano leaves contain flavonoids, saponins, tannins, and steroids/terpenoids, which have anti-diabetic activity [10], [11]. A study in diabetic mice demonstrated that blood glucose levels were reduced by 44.47% after a 10-day treatment with n-hexane-extracted puguntano [12].

Tannins promote PI3K and p38 MAPK activity and GLUT-4 translocation [13], while flavonoids promote GLUT-4 translocation [9], terpenoids increase GLUT-4 expression and translocation through *proliferator-activated receptor gamma* (PPAR- γ) activation [14], [15], [16], and saponins increase GLUT-4 expression [17], [18]. Also, a previous study showed that quercetin from berry extract with flavonoid compound increases insulin receptor substrate 1 (IRS1), IRS2, AKT, p38 MAPK, adenosine monophosphate-activated protein kinase (AMPK) and GLUT-4 expression in skeletal muscle cells [19]. Furthermore, Lindarto et al., Reported that insulin resistance is ameliorated in newly diagnosed T2DM patients after treatment with puguntano leaf extract for 12 weeks, illustrated by the significant reduction in fasting blood glucose (FBG) levels, homeostasis model assessment-insulin resistance (HOMA-IR), and glycated haemoglobin (HbA1c) [20].

The present study aimed to determine the

effect of puguntano leaf extract (*Curanga feel-terrae* Merr.) on p38 MAPK levels and GLUT-4 expression in a rat model of T2DM.

Material and Methods

Forty-eight male 8-week-old Wistar rats weighing 180-200 g were housed in stainless steel cages under environmentally controlled conditions. The ambient temperature was 22-25°C, and the light/dark cycle was 12/12 hours. The animals had free access to water and standard diet. After 3 days' acclimatisation, the rats commenced consumption of a high-fat diet (HFD), which continued for 5 weeks and was followed by two intraperitoneal injections of low-dose streptozotocin (STZ; 30 mg/kg), 1 week apart [21]. STZ was dissolved in 50 mM sodium citrate solution (pH 4.5) containing 150 mM NaCl [22]. After the induction of diabetes using HFD and STZ, fasting blood glucose (FBG) levels were measured in the blood from the tail vein using a glucometer. Rats with FBG level > 200 mg/dL were considered to be diabetic [21].

Diabetic rats were then randomly divided into control and treatment groups, each containing 24 rats. The treatment group was administered with an ethanolic extract of puguntano leaves in carboxyl methyl cellulose-Na (CMC-Na; 0.5% solution; 200 mg/kg/day) using an orogastric cannula for 10 days. The extract was prepared by maceration in Department of Biological Pharmacy, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia [23].

At the end of the experiment, blood was obtained from the left ventricle, left undisturbed at room temperature for 15–30 min, then centrifuged at 1-2,000 \times g for 10 min. FBG levels were determined using spectrophotometry and fasting insulin using sandwich ELISA. The rats were euthanised using ketamine and decapitated, and then gastrocnemius muscles were dissected for examination of p38 MAPK levels and GLUT-4 expression.

p38 MAPK levels was evaluated from a slice of muscle that was placed in round bottom microfuge tube sand than either snap frozen or kept on ice for immediate homogenization. For a ~5 mg piece of tissue, ~300 μ L complete extraction buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and 0.5% Sodium deoxycholate) was added to the tube and homogenized using an electric homogenizer. The blade was rinsed twice using 300 μ L complete extraction buffer; then the homogenate was agitated for 2 hr at 4°C and centrifuged for 20 min at 13,000 x rpm at 4°C then the supernatant was transferred to a fresh, chilled tube and store samples at -80°C. The

cell extraction was supplemented with phosphatase, protease inhibitor cocktails and PMSF to 1 mM, immediately before use. After thawing, samples were centrifuged before use at 10,000 rpm for 5 min at 4°C to remove any precipitate.

GLUT-4 expression was evaluated in paraffin-embedded sections of rat skeletal muscle tissue. Four-millimetre-thick paraffin sections were dewaxed, rehydrated, and microwaved for 10 minutes. The endogenous peroxidase activity of the investigated specimens was blocked using 3% H₂O₂ for 10 minutes, followed by 25 minutes washing with phosphate-buffered saline (PBS). The tissue sections were incubated with normal rabbit serum for 10 minutes, and then the slides were incubated at room temperature with rabbit polyclonal anti-Glucose Transporter GLUT-4 rat antibody (b33780). Sections were then washed with PBS and incubated with a secondary antibody goat anti-rabbit polyclonal IgG for 30 minutes, washed twice with PBS, counterstained with haematoxylin, and mounted using DPX. A positive signal for GLUT-4 in muscle tissue was semi-quantitatively estimated by recording the distribution of positively stained cells and the intensity of the staining at the plasma membrane. Cell counting was performed using a light binocular microscope, and the data were presented as immunohisto score.

This experimental protocol was approved by the Institutional Ethics Committee of Universitas Sumatera Utara, Medan, Indonesia (Reference 42/TGL/KPEK FK USU-RSUP HAM/2018).

Biochemical analysis

STZ was purchased from Sigma Aldrich (Munich, Germany). FBG was measured using a commercially available enzymatic kit. Fasting insulin and p38 MAPK levels were determined using commercial kits supplied by Qayeebio (China). GLUT-4 expression was determined using a kit supplied by Abcam (Cambridge, UK). FBG, fasting insulin, p38 MAPK levels were quantified in the Molecular Genetics Laboratory of the Medical Faculty of Universitas Padjajaran, and GLUT-4 expression was determined in the Immunopathology Laboratory of the Department of Anatomical Pathology, Hasan Sadikin Hospital Bandung. The HOMA-IR equation, which estimates the degree of insulin resistance using fasting insulin and glucose levels, was used as previously described [24].

Statistical Analysis

Values were expressed as the median (minimum-maximum). Data were analysed using the Wilcoxon test in SPSS version 22.0 to identify statistically significant differences between groups, which were accepted when p < 0.05.

Results

As shown in Table 1, FBG, fasting insulin, and HOMA-IR were significantly lower in the treatment group than in the control group.

Table 1: FBG, fasting insulin and HOMA-IR in the control and treatment group

Variable	Group		P
	Control (n = 24) Med (min-max)	Treatment (n = 24) Med (min-max)	
FBG (mg/dl)	384 (207-490)	122 (95-213)	0.001*
Fasting insulin (µU/ml)	56.56 (49.63-73.67)	51.31 (47.77-59.00)	0.001*
HOMA-IR	2.77 (2.60-3.71)	0.77 (0.59-1.29)	0.001*

Wilcoxon test. *Significant if p < 0.05.

As shown in Table 2, p38 MAPK and GLUT-4 protein expression were higher in the treatment group than in the control group.

Table 2: p38 MAPK levels and GLUT-4 expression in the control and treatment group

Variable	Group		p
	Control (n = 24) Med (min-max)	Treatment (n = 24) Med (min-max)	
p38MAPK (ng/mL)	19.70 (17.37-27.34)	23.24 (17.74-34.41)	0.001*
GLUT-4 (score)	2 (1-6)	4 (2-9)	0.001*

Wilcoxon test. *Significant if p < 0.05.

Figure 1 showed the effects of a puguntano leaves extract on the histological features of GLUT-4 expression in T2DM diabetic rat muscle.

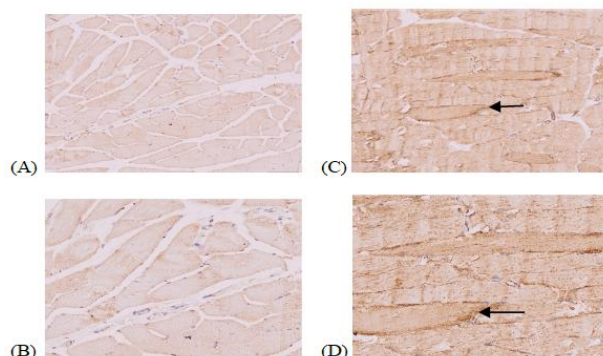


Figure 1. Effects of a puguntano leaves extract on the histological features of skeletal muscle in a rat model of T2DM. GLUT-4 expression in the control rats (A and C; 100X and 200X magnification, respectively) and treated rats (B and D; 100X and 200X magnification, respectively) demonstrating higher GLUT-4 expression in the plasma membrane of T2DM rats that had been treated with puguntano extract (Arrow)

Discussion

The rat model of diabetes induced by HFD feeding and low-dose STZ closely simulates the natural pathogenesis of T2DM and is widely used in studies of the efficacy of anti-diabetic drugs. Induction with HFD/STZ will exhibit hyperglycemia and insulin

resistance [25]. This study is the first study to evaluate the effect of puguntano leaf extract (*Curanga feell-terrae* Merr.) on p38 MAPK levels and GLUT-4 expression in such a rat model of T2DM.

Insulin binding to the α -subunits of the insulin receptor (IR) increases the receptor β -subunit tyrosine kinase activity, which results in phosphorylation of IR substrates (IRS; IRS-1 and IRS-2 in skeletal muscle). These IRS proteins then interact with the specific SH2 domains of downstream molecules, including PI3K, Grb2, and phosphotyrosine phosphatase (SHP2). IRS-1-Grb2 binding initiates a cascade that activates the protein Ras and MAPK, and consequently nuclear transcription factors. IRS-PI3K binding generates phospholipids that activate further downstream kinases and ultimately induced physiologic response such as glucose transport, and protein and glycogen synthesis [26], [27]. These kinases are the serine/threonine kinases [AKT/protein kinase B (PKB) and protein kinase C (PKC)]. Phosphorylation of AKT initiates the translocation of GLUT4 from its intracellular storage location to the surface of the cell to facilitate glucose transport into the cell [28], [29].

Fasting insulin concentration is a significant indicator of insulin resistance and is increased in obesity [24]. In this study, FBG, fasting insulin levels, and HOMA-IR were significantly lower in the treatment group than in the control group. This may be explained by an effect of one or more secondary metabolites the tannins, flavonoids, triterpenoids, and saponins present in puguntano leaf extract to insulin sensitivity. The results of the present study are consistent with those of Lindarto et al., who showed the anti hyperglycemic and insulin-sensitizing effect of puguntano leaf extract in decreasing FBG and HOMA-IR in newly diagnosed T2DM patients [20].

Muscle p38 MAPK levels and GLUT-4 expression were significantly higher in the treatment group than in the control group in this study. These effects of puguntano are similar to those reported for quercetin, a citrus flavonoids present in berry extract that caused increases in p38 MAPK and GLUT-4 expression in L6 myotubes. Indeed, quercetin was reported to have anti-diabetic effects through activation of both the PI3K/AKT and MAPK pathways, inducing glucose uptake through the increasing of GLUT-4 expression and translocation [19].

The significant increase in GLUT-4 expression noted in the treatment group was also consistent with that in previous studies of other plant extracts containing tannins, flavonoids, triteropenoids, and/or saponins. A study by Xiong et al., showed that *Entada phaseoloides* (L.) Merr. With the major secondary metabolite triterpenoid saponin Entagenic acid (EA) promoted glucose uptake into the skeletal muscle of T2DM rats by enhancing the translocation and expression of GLUT-4 [5]. An ethanolic extract of *Vernonia amygdalina* Del. (VA) which contains a high concentration of flavonoid polyphenols also caused a

significant increase in GLUT-4 expression (24%) and translocation (35.7%) to the plasma membrane of skeletal muscle in diabetic treatment group [30]. Furthermore, there was higher GLUT-4 expression after the administration of other herbal products containing triterpenoids saponins [31], flavonoids [32], [33], and tannins, flavonoids and triterpenoids [34].

In conclusion, puguntano leaf extract improved post-receptor insulin signalling by increasing p38 MAPK levels and GLUT-4 expression in a rat model of T2DM. Further studies should be undertaken to establish whether it may represent a novel therapy for T2DM in people.

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