

Expression Profiling Of Diabetes-Related Genes In Streptozotocin-induced Rat After Treatment With Herbal Mixture Extract

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Abstract

Diabetes mellitus type 2 is a metabolic disorder disease that is caused mainly by insulin resistance and relatively lack of insulin being produced which eventually will lead to the hyperglycemia. Since the occurrence of diabetes mellitus type 2 have increased markedly, it has become a worldwide problem with obesity and bad lifestyle as the main factor contributing to diabetes mellitus type 2. Synthetic drugs such as metformin are widely used to lower down glucose level and to reduce the occurrence of diabetes. However, herbs also have ability to control the occurrence of diabetes naturally. Recent study shows that the mixture of Sambung Nyawa (*Gynura procumbens*) and Dukung Anak (*Phyllanthus watsonii*) extract was capable to lower down the glucose level in streptozotocin-induced rat after 8 weeks of treatment. Apart from it, the herbal extract mixture was also found to positively regulate a number of genes related to rat's diabetes. The genes that were positively regulated by herbal extract treatment were involved in insulin resistance, signaling of hormone, vascular response and also genes that involved in glucose and lipid metabolism pathways.

Keywords: Gene expression, herbal, rat, diabetes

Introduction

Many of Malaysian consume excessive amount of sugar in their daily diet. This unhealthy lifestyle increases the risk of diabetes which is not only a major disease in Malaysia but also a worldwide problem. The prevalence data from World Health Organization (WHO) indicate that 942,000 Malaysian suffered with diabetes in 2000 and this number estimated to increase to 2,479,000 in 2030. There are lots of chemical agents available to control and to treat diabetic patients, but total recovery from diabetes has not been reported up to this date. Alternative to these synthetic agents, plants provide a potential source of hypoglycemic drugs and are widely used in several traditional systems of medicine to prevent and to reduce the occurrence of diabetes. The effects of these plants may delay the development of diabetes complications and correct the metabolic abnormalities using variety of mechanisms (Bnouham et al., 2006).

Phyllanthus sp. or locally known as Dukung Anak was reported to have anti-hyperglycemic activity (Okoli et al. 2011, Shetti et al. 2012). *Phyllanthus niruri* extract was reported to reduce blood glucose,

suppressed postprandial rise in blood glucose following a glucose meal, reduced hemoglobin glycation and increased absolute and relative weights as well as glycogen content of liver in diabetic rats. Treatment with the *Phyllanthus niruri* extract also ameliorated the decrease in body weights caused by the diabetic disease (Okoli et al. 2011). In the research conducted on *Phyllanthus amarus*, the ethanol extract exhibited anti-diabetic or hypoglycemic activity on alloxan induced diabetic mice due to the enhancement of glucose utilization by peripheral which eventually correcting the impaired liver or kidney glycolysis and also suppression of its gluconeogenic activity similar to insulin (Shetti et al. 2012). Based on research conducted in MARDI, the Dukung Anak have high content of geraniin. Geraniin was reported to have anti-hyperglycemic activity (Palanisamy et al. 2011).

Gynura procumbens or locally known as Sambung Nyawa has been reported to successfully control and reduce risk of diabetes (Rosidah et al., 2008). Phytochemicals such as phenolic compounds (Rasidah et al., 2008), terpenoids (Siti Pauliena, 2006) and plant membrane lipids (Saengsai, 2003) have been discovered in *G. procumbens*. Based on research which

have been conducted in MARDI, *G. procumbens* extract contains high level of dicaffeoylquinic acid and the treatment of *G. procumbens* extract on streptozotocin induced rat reduce the high glucose content in rat blood. It was reported that high content of dicaffeoylquinic acid in artichoke have anti-hyperglycemic activity (Heidarian et al. 2011). These bioactive compounds in Dukung Anak and Sambung Nyawa are believed to act synergistically and reduce the risk or occurrence of diabetes.

However, many research on the effectiveness of *Phyllanthus spp.* and *G. procumbens* was based on biochemicals test which was been carried out on animal models and human cell cultures (Saengsai 2003, Siti Pauliena 2006, Rasidah et al., 2008, Heidarian et al. 2011, Okoli et al. 2011, Shetti et al. 2012). Up to date, there is no study at molecular level of mode of action of *Phyllanthus spp.* and *G. procumbens* bioactive compound. Although biochemicals test is able to validate the effectiveness of herbs bioactive compound, the addition of more validation method such as using transcriptomics method will strengthen the scientific prove of herbal medication. Through the advancement of human genome project, genes involve in the pathway of diabetes in both human and animal model are available. By utilizing bioinformatics tools, many scientific evidence which have been carried out by all over the world on the genes related/involved in diabetes mellitus can be gathered together to obtain important relevant information for pathways analysis.

Up to date, genes involved in diabetes are grouped into six functional categories which are receptors, transporter and channels genes, nuclear receptors genes, metabolic enzymes genes, secreted factors genes, signal transduction proteins genes and transcription factor genes. These genes involved in the onset, development and progression of diabetes. The availability of these genes information are useful for gene expression analysis of the effect *Phyllanthus spp.* and *G. procumbens* bioactive compound in reducing the occurrence of diabetes. The advantages of identifying and validating the efficiency of anti-diabetes bioactive compound using molecular approach is the ability to screen highthroughputly in a shorter period of time.

Materials & Methods

Induction of diabetic rat using streptozotocin

Normal healthy Sprague-Dawley male rats (250-300g) were acclimatized for 1 week with standard pellet diet and water given *ad libitum*. After 1 week, diabetic rat was induced by using streptozotocin (50mg/kg) which is dissolved in citrate buffer (pH 4.3) immediately prior to use. The solution was injected intraperitoneally

according to the streptozotocin dose. The blood glucose level was recorded per weekly for 6 weeks. Rats showing blood glucose more than 15 mmol/L is considered diabetic. The control group study does not involve in the induction of diabetes. At the end of 6 weeks, blood was taken for serum analysis without killing the rats.

Treatment of diabetic rat with herbal formulation mixture

After 6 weeks of induction with 50 mg/kg of streptozotocin, the selected diabetic rats were divided into 3 groups which are diabetic rats only induced by streptozotocin (Group 1), diabetic rats with the treatment of 10 mg/kg metformin (Group 2) and diabetic rats with the treatment of 700mg/kg herbal formulation mixture (Group 3). The normal rats was used as a control for this study. The treatment was conducted for 8 weeks. The blood glucose reading was recorded per weekly until end of the treatment. After 8 weeks of treatment, rats were dissected to collect blood for transcriptomic analysis.

Total RNA isolation & cDNA synthesis

10 blood samples from each groups of rat were used for total RNA extraction. Approximately 1 ml of Rat blood was used for RNA extraction using QIAamp® RNA Blood Mini Kit (Qiagen, Germany). The purity and concentration of total RNA was measured using Nanodrop. The integrity of Total RNA was checked using electrophoresis of 1% Agarose gel. Only total RNA with OD A_{260/280} around 1.9-2.1 and OD A_{260/230} >1.7 was used for analysis of PCR Array.

Gene expression profiling using PCR Array

Total RNA isolated from blood sample was converted to cDNA using RT² First Strand Kit (Qiagen, Germany). 2 µg of total RNA for each sample were used for cDNA synthesis and cDNAs obtained were kept in -20°C freezer. Prior to use, the cDNA sample was diluted 5x and was mixed with PCR Array master mix before being disbursed into PCR Array plate. For PCR Array analysis, every sample is consisting of 3 replicate of experiments. PCR Array that being used are for Rat Diabetes RT² Profiler™ PCR Array (Qiagen, Germany). PCR Array containing 84 genes related to diabetes with 5 housekeeping genes were used as internal control to normalize gene expression. Together with this PCR Array plate, MGDC gene was used to determine whether any genomic DNA contamination occurred and PPC amplification for positive PCR control. Data obtained from this analysis were using $\Delta\Delta^{Cp}$ Method to analysis gene expression level between

control and treatment plate.

Bioinformatic Analysis

Data obtained from PCR Array analysis were uploaded to the PCR Array data analysis website (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>) to analysis the gene expression level between control and treatment plate. Genes related to diabetes that was positively regulated to the herbal treatments were further analysed using bioinformatics software such as Pathways Studio software and KEGG pathways database in order to elucidate the mode of action of herbal treatments.

Data analysis and results

The herbal mixture formulation treatment was successfully conducted on diabetic rat for 8 weeks period. The blood was successfully harvested from treated diabetic rat and total RNA was obtained from it as in figure 1.0. The total RNA obtained from rat's blood was around 0.1 µg/µl to 0.5 µg/µl depends on the amount of blood sample used. The quality of total RNA was also found in good condition with the A_{280/260} and A_{260/230} was in the acceptance range to be used for qPCR analysis.

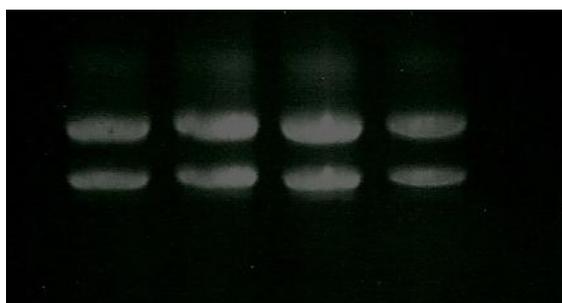


Figure 1.0: 1.0% gel electrophoresis of total RNA isolated from rat's whole blood

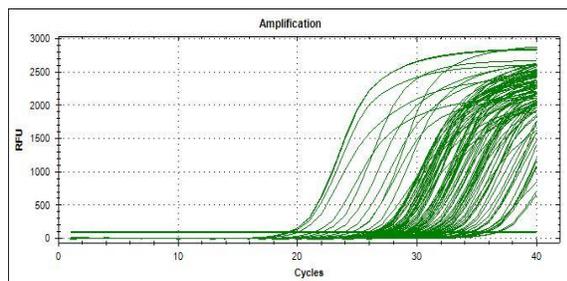


Figure 2.0: Amplification plot of PCR Array for genes related to diabetes after 40 cycles.

Rat Diabetes RT² Profiler™ PCR Array amplification plot was obtained after the qPCR analysis

was performed in thermocycler machine for 40 cycles. The C_p value for calculation of gene expression was obtained from generated amplification plot (Figure 2.0). The C_p value data was arranged according to the format given by the manufacture and was uploaded into online database in order to calculate the expression value of gene related to diabetes between treatment and negative control group. Based on the gene expression profiling obtained from PCR Array analysis, 24 genes related to diabetes were positively regulated in order to correct the abnormality which caused the occurrence of diabetes. These genes mainly involved in insulin resistance, signaling of hormone, vascular response and also genes that involved in glucose and lipid metabolism. The genes that are positively regulated by herbal extract are: *CCL5, CEBCA, DPP4, ENPP1, G6PC, GCG, HMOXI, ICAMI, IKBKB, IL4RA, MAPK14, MAPK8, NFKB1, PARP1, PIK3CD, PTPN1, PYGL, RETN, SNAP23, TGFBI, TNF, TNFRSF1A, TRIB3, UCP2* and *ABCC8*.

Discussions

Comparison profiling of diabetes related genes in PCR Array plate between untreated diabetic rat sample and diabetic rat treated with herbal mixture formulation show that 25 genes were found to regulate positively when herbal treatment were given (Table 1.0). Based on the bioinformatics analysis, it was found that the herbal formulation treatment positively affected 4 main pathways that were involved in diabetes mellitus (Figure 3.0). The first pathway is insuling signaling pathways where the genes such as *GCG, HMOXI, PIK3CD, PTPN1, PYGL, TRIB3* and *ABCC8* were positively regulated by the herbal treatment. The second pathway that was affected by herbal formulation treatment is glucose metabolism pathways. In glucose metabolism pathways, genes such as *DPP4, ENPP1, G6PC, UCP2* were positively regulated after herbal treatment.

Table 1.0: Positively regulated diabetes related genes in diabetic rat treated with herbal formulation mixture when compared to untreated diabetic rat

Genes	Description
<i>ABCC8</i>	<i>ATP-binding cassette transporter sub-family C member 8</i>
<i>CCL5</i>	<i>Chemocine(C-C Motif) ligand 5</i>
<i>CEBCA</i>	<i>CCAAT/enhancer binding protein (C/EBP) alpha</i>
<i>DPP4</i>	<i>Dipeptidylpeptidase</i>
<i>ENPP1</i>	<i>Ectonucleotide pyrophosphatase/phosphodiesterase 1</i>
<i>G6PC</i>	<i>Glucose-6-phosphatase</i>

<i>GCG</i>	<i>Glucagon</i>
<i>HMOX1</i>	<i>Heme oxygenase 1</i>
<i>ICAM1</i>	<i>Intercellular adhesion molecule 1</i>
<i>IKBKB</i>	<i>Inhibitor of kappa light polypeptide gene enhancer in β-cell, kinase beta</i>
<i>IL4RA</i>	<i>Interleukin 4 receptor, alpha</i>
<i>MAPK14</i>	<i>Mitogen activated protein kinase 14</i>
<i>MAPK8</i>	<i>Mitogen activated protein kinase 8</i>
<i>NFKB1</i>	<i>Nuclear factor of kappa light polypeptide gene enhancer in β-cells 1</i>
<i>PARP1</i>	<i>Poly (ADP-ribose) polymerase 1</i>
<i>PIK3CD</i>	<i>Phosphoinositide-kinase, catalytic, delta polypeptide</i>
<i>PTPNI</i>	<i>Protein tyrosine phosphatase, non-receptor type 1</i>
<i>PYGL</i>	<i>Phosphorylase, glycogen, liver</i>
<i>RETN</i>	<i>Resistin</i>
<i>SNAP23</i>	<i>Synaptosomal-associated protein 23</i>
<i>TGFB1</i>	<i>Transforming growth factor, beta 1</i>
<i>TNF</i>	<i>Tumor necrosis factor (TNF superfamily, member 2)</i>
<i>TNFRSF1A</i>	<i>Tumor necrosis factor receptor superfamily, member 1a</i>
<i>TRIB3</i>	<i>Tribbles homolog 3</i>
<i>UCP2</i>	<i>Uncoupling protein 2</i>

The third pathway that affected by the herbal treatment is Adipocytokine pathways with genes such as *IKBKB*, *MAPK8*, *NFKB1*, *RETN* and *TNF* were found to express at normal level when compared to the expression pattern of normal rat. While the genes such as *CEBPA*, *MAPK14*, and *TGFB1* which are involved in Glucocorticoid pathway. Other genes that are positively regulated by herbal treatment are such as *CCL5*, *ICAM1*, *IL4RA*, *PARP1*, *SNAP23* and *TNFRSF1A* which are involved in others pathways such as cell toxicity, vascular response and membrane trafficking.

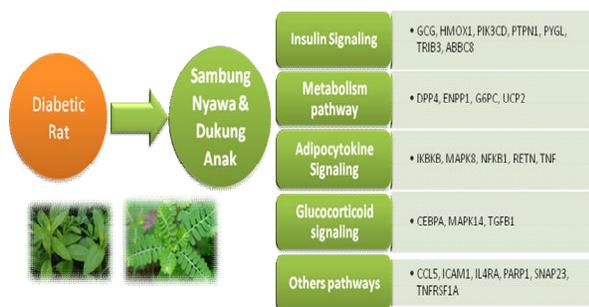


Figure 3.0: Genes related to diabetes affected by herbal mixture formulation treatment.

Conclusions

As conclusion, the herbal formulation mixture was found to affect a few pathways related to diabetic in order to correct the abnormality of metabolic regulation that responsible for the development of diabetes disease.

Acknowledgment

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