Differential Expression Analysis of Diabetic Related Genes in Streptozotocin-Induced Diabetic Rat in Response to *Abelmoschus Esculentus* Treatment:A Research Framework

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Abstract— **Diabetes is characterized by excessive blood sugar due to body's failure to produce insulin or the consequence of insulin resistance. Nowadays, human suffering not only on the disease itself but also includes diabetes-related complications. All these abnormality are significantly important because of their effects on the liver function. Therefore this paper will provides an overview of the research framework to investigate the differential expression analysis of liver tissue in streptozotocin-induced diabetic rat in response to *Abelmoschus esculentus* (AE) treatment. The hypoglycemic effects of water extract prepared from the fruit of AE will be studied in diabetic rats (streptozotocin induced). All animals will be randomly divided into three group; normal healthy group (N group), streptozotocin–induced diabetic group (STZ group) and AE-treated diabetic group (AE group). Oral application of AE at doses of 100, 150 and 200 mg/kg body weight will be given to AE-treated diabetic group by single and repeated oral administration. Rats liver from these three groups will then subjected to RNA extraction for gene expression analysis. The differential expression study will be carried out by using real time RT-PCR method. Four diabetes-specific genes of interest (carboxylesterase 2, stearoyl-Coenzyme A desaturase 1, insulin-like growth factor 1 and insulin-like growth factor binding protein 2 binding protein) are chosen and the expression level of these genes will be examined quantitatively. The abnormal expression of genes in STZ group will be rescued by the AE therapy. The expected findings from both *in vivo* and molecular studies may reveal the anti-diabetic properties of the AE and suggest that the plant extract may be useful for the management of the disease. This paper will provide the overview of the research framework and giving insight of the experimental procedure to be implemented.**

   Keywords-diabetes mellitus, abelmoschus esculentus, gene expression, stretozotocin

I.   INTRODUCTION

Diabetes mellitus (DM) is a common metabolic disease characterized by the increased circulating glucose concentrations. It is correlated with abnormalities in variety of microvascular, macrovascular, neurologic and infectious complications including carbohydrate, fat, and protein metabolism. Type 2 DM begins with a period of insulin resistance with increased pancreatic insulin secretion. As the disease progresses, pancreatic function weaken and as a result, insulin levels fail to keep up with the body requirements [1].

Currently DM is one of the most costly and burdensome chronic diseases and is a condition that is increasing in epidemic proportions throughout the world. It is reported by WHO, diabetes affects about 5% of the global population [2]. The management of diabetes without any side effects is still a challenge to the medical system. It is commonly known that the treatment for diabetes is relatively limited with significant side effects. There is growing interest in the use of natural health products as an alternative approach to current medications. Plant sources has become a target to explore new drugs and in searching biologically active compounds [3].

The streptozotocin-induced diabetic rat is still considered as an important means for the pathophysiology and pharmacology studies of diabetes mellitus. Some of the research has done using diabetic rat to investigate the anti-diabetic properties are mentioned in the literature. The next section discusses the literature review, research framework, expected results and conclusions.

II.   LITERATURE REVIEW

This sections provides a summary of the literature review on diabetes mellitus, AE, anti-diabetic properties on medicinal plants using diabetic rats, AE used as traditional remedy in different countries for different illness control, nutritional healing of AE and literature on various research on few of the bioactive properties of AE.

A.   *Diabetes Mellitus (DM)*

Diabetes mellitus (DM) is the most challenging metabolic disorder as it cannot be cured but needs only to be managed. International Diabetes Federation (IDF) reported that type 2 diabetes influence 246 million citizens and expected to increase to astounding 380 million by 2025 [cited in 5]. In Malaysia, it is reported in 2010, that the diabetic sufferers are approximately 3.4 million [4]. By far, Asia was recognized as having the potential increase with 2.5 to 3 times more common diabetes [4]. Hence, by 2010, Asia was expected to have 138 million diabetic patients [4].

Peripheral insulin resistance is a key feature of type 2 DM and the consequences from a combination of sedentary

lifestyle, unhealthy dietary habits, and genetic predisposition [6]. Insulin resistance is also implicated in a numeral of life threatening disorders collectively known as the metabolic syndrome [6]. Consumption of the indigenous plant materials are in use for the management of DM since long and differ from place to place.

B. Abelmoschus Esculentus (AE)

According to Maganha *et. al* (2009) [7] AE, known as lady's finger, is a very poorly studied species. It is reported that the biological activity in this species was the *in vitro* antioxidant potential [8]. The major antioxidant molecules were identified to be *quercetin* derivatives and *epigallocatechin* [8]. There is lacking scientific reported study on these plant properties despite its wide usage as medicinal plant. Therefore, the main objective of this study is to investigate the underlying molecular mechanism on the natural hypoglycemic substances effect in AE.

C. Anti-Diabetic Properties on Medicinal Plants using Diabetic Rats.

TABLE I.   RESEARCH ON ANTI-DIABETIC PROPERTIES OF MEDICINAL PLANTS FROM DIFFERENT COUNTRIES USING DIABETIC RATS

Herbs or medicinal plants	Country/Region	Author
Lupinus albus, L. Cymbopogon proximus,Zygophyllum coccineumL.	Egypt	[11]
M charantia, A sativum, A indica, O sanctum,etc	India	[12]
Cuminum cyminum		[14]
Momordica charantia		[15]
Gymnema montanum		[18]
Cinnamonum zeylanicum		[29]
Aerva lanata		[30]
Trigonella foenum-graecum Linn Ocimum sanctum Linn Pterocarpus marsupium Linn		[31]
Ramulus mori	China	[13]
Gymnema sylvestre	Japan	[16]
Clausena anisata	South Africa	[17]
Artemisia campestris	Tunisia	[19]
Retama raetam	Saudi Arabia	[21]
Loranthus bengwensis L.	Nigeria	[22]
Bauhinia forficate	Brazil	[23]
Eugenia aromaticum	Asia	[24]
Morus alba	Thailand	[25]
Rubus fructicosis L.	Africa	[26]
Globularia alypum L.		[26]
Malmea depressa	Mexico	[27]
Eucalyptus globulus	South America & Africa	[28]
Dryopteris fragrans,Filix maris	Mongolia	[32]

Numerous research had been conducted that revealed anti-diabetic properties of medicinal plants from different countries using diabetic rats. The following Table I provides a summary of the literature on the anti-diabetic properties investigation from respective countries.

D. AE Used As Traditional Remedy In Different Countries For Different Illness Control

TABLE II.   AE USED AS TRADITIONAL REMEDY IN DIFFERENT COUNTRIES FOR DIFFERENT ILLNESS CONTROL

Illness control	Country	Author
Stomach pain	Africa	[33]
Antiasthma	Latin America	[34]
Hypoglycemic effect	India	[35]
Stomach irritation	Sri Lanka	[36]
Stomach irritation & inflammation	Asian	[37]

Table II presents the literature on AE used as traditional remedy in different countries for different illness control.

E. Nutritional Healing of AE

TABLE III.   NUTRITIONAL HEALING PROPERTIOS OF AE

Healing properties	Author
Anti-ulcer food	[38]
Anticancer, reduced heart attack, lower blood cholesterol, relieve intestinal disorder, relieve inflammation of the colon, relieve diverticulitis, relieve stomach ulcer	[39]
Neutralize acid, lubricate large intestine, treatment of lung inflammation, treatment of irritable bowel, keep joints limber, treatment of sore throats, heal burn soothe poison, soothe psoriasis	[40]

Table III presents the literature on few of the nutritional healing properties of AE

F. Research on Few Of The Bioactive Properties Of AE

Table IV presents the literature on research on few of the bioactive properties of AE

TABLE IV.   RESEARCH ON BIOACTIVE PROPERTIES OF *ABELMOSCHUS ESCULENTUS*

Bioactive Properties	Author
Antioxidative effect	[41]
Antibacteria effect	[42],[43]
Hypoglycemic effect	[44]
Inhibitory activities of mucilages & gums	[45]

III.   RESEARCH FRAMEWORK

The research framework of this study comprise of; preparation of the aqueous extract, animal experiments, isolation of total RNA, real time reverse transcriptase-PCR (RT-PCR), gel electrophoresis.
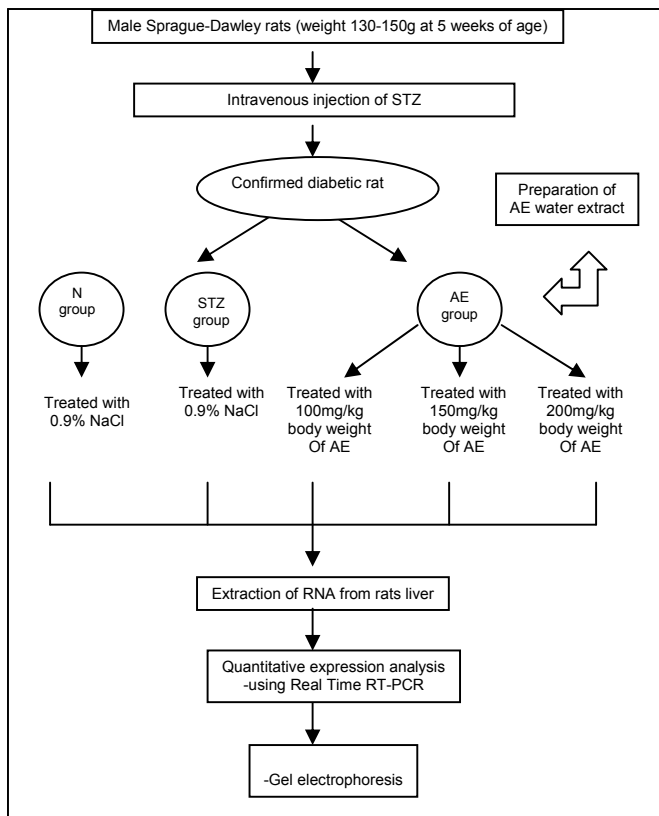
Figure 1.   Research Framework

The following provides the explanation of each of the stages:

A.   Preparation Of The Aqueous Extract

The plant in this study will be collected in its natural habitats and botanically determined by Forestry Research Institute Malaysia (FRIM), Malaysia. The whole plant will be ground and 50 g of powder mixed with 500 ml of distilled water (10%). The mixture is heated and boiled under reflux for 30 min. The decoction obtained is centrifuged, filtered, frozen at −20 °C, lyophilised and used without further purification.

B.   Animal Experiments

Experiments will be carried out in adult male Sprague-Dawley rats weighing from 130 to 150 g at age of 5 weeks according to standard procedure. Animals house under standard environmental conditions *(23±1∘C,   55±5% humidity and a 12-h light/dark cycle)* and maintained with free access to water and a standard laboratory diet. Experimental rats will be induced by intravenous injection of streptozotocin (STZ) (Sigma, St. Louis, MO, USA) into the tail vein (dose 50 mg/kg body weight). STZ is dissolved in 0.1M cold sodium citrate buffer, pH 4.5 and the control rats are injected with vehicle alone. Diabetes will be verified by evaluating blood glucose levels and diabetic rats are confirmed if blood glucose level greater than 300mg/dl (16.7 mmol/*l*). All the animals are randomly divided into three groups: (*n*=7 per group).

Group 1: Normal healthy control group (N group), normal rats received 0.9% NaCl solution.
Group 2: STZ-induced diabetic group (STZ group), diabetic rats treated with 0.9% NaCl solution
Group 3: AE-treated diabetic group (AE group), diabetic rats treated with AE at three different level (100, 150 and 200mg/kg body weight) and blood glucose levels determine in fasted rats, every 4 to 6 hrs, after 2 and 4 days, 1 and 2 weeks of single or repeated oral administration.

C.   Isolation of Total RNA

Total RNA from frozen liver tissue will be isolated with the RNeasy Mini Kit (Qiagen, Stanford, CA). Samples will be processed following the manufacturer's directions. At the last step, the RNA will be eluted with 50 μl of RNase-free water. Quality of the RNA will be assessed using Agilent Bioanalyzer System.

D.   Real Time Reverse Transcriptase-PCR (RT-PCR)

The assay performs using a one-step RT-PCR kit (Qiagen, Germany). A set of primers and a probe will be selected for the in-house real-time RT-PCR assay, forward, reverse, and TaqMan probe. The in-house real-time TaqMan assay will be carried out using QuantiTect Probe RT-PCR kit (Qiagen,Germany). The reaction contained 5 μl of RNA, 0.6μM of each primer, 0.2 μM of the TaqMan probe. An Appliee Biosystem real time-PCR machine is used with the following thermal steps: reverse transcription 30 min, initial denaturation, followed by 40 cycles of denaturation, annealing for , and extension

E.   Gel Electrophoresis

Assessment of PCR products will be carried out using Agilent Bionalayzer system. The PCR product will be visualized as a single compact band with expected size.

IV.   EXPECTED RESULTS

The following subsections provide insights on the expected results from the experiments.

A.   Preparation Of The Water Extract

In this study, a water extract use as a method of choice in order to follow similar traditional preparation had done in many countries. Moreover it also used as a preliminary study before searching on the pure compound.

B.   Animal Experiments

A comparison of the glucose levels from three different groups will be studied. STZ induce diabetic rat should significantly elevated the blood glucose levels after 3 months compared with rats injected with vehicle alone (control group). These observations are according to Verspohl (2002) [46] for the development of a type 2 diabetic model. In the diabetic rat model, the extracts will show significant hypoglycemic effect and the effect of the administered extract is dose dependant. It is expected that the lower dose (100 mg/kg) of the AE only produced a statistical significant

effect less than the higher dose (150 mg/kg), after its administration. The data obtained from this study will be the first documented data demonstrated the appropriate doses that can be traditionally used for treating diabetic people. In addition, the result should not show any toxicity and mortality occur to the control group. This is based on the fact that there is no data reported of toxicity occur among its consumer.

C. Molecular Studies

Gene expression can be assessed by measuring the quantity of the final product for instance, the protein. However in this study, measuring RNA level is chosen because it is more efficient with the current technologies and easy access of information. Moreover, available proteomic technologies are generally lower throughput and more challenging.

It is critically important to have a good quality of RNA in any of expression study. To ensure the reliability of the RNA samples, RNA assessment will be carried out using Agilent Bioanalyzer system. Successful isolation technique yields good quality of RNA. The intact total RNA sample will show distinct 18S and 28S subunit spikes, with 2:1ratio (28:18S).

In this study, real-time RT-PCR is chosen because it enables us to view PCR amplification in each cycle in faster and easiest way. Compare to other quantitative RT-PCR, real time RT-PCR requires shorter development, normally less variable and more reproducible. Protocol chose for this method is using Taq-Man assay. The advantage of this method is more specific because of the probe designed and will also avoid the primer dimmer.

In studies of target gene expression by RT-PCR, the use of internal reference genes is required to control for RNA quality, reverse transcription efficiency and overall transcriptional activity in samples. Preferably, internal standards should be constitutively expressed by all cell types independent of experimental conditions and they should not be affected by any disease. For normalization, housekeeping genes of β-actin is employed. From this study, the expected real time RT-PCR product will be confirmed by post-PCR gel analysis. This additional step will be carried out as to make sure the amplified product within the expected size

Analysis of gene profiling study on STZ induce diabetic rat did by Hwang et al, 2009, [47] demonstrated many changes occurred in their gene expression. More than a half of gene analyzed (565 from total 835 genes) showing two-fold differences compared to the control. The differentially expressed genes involved in carbohydrate, amino acid metabolism, immunity and defense, lipid, fatty acid , steroid metabolism and signal transduction [47]. In this study, four specific diabetes genes will be chosen to present the hypoglycaemic effect of the extract. Treatment with AE may show significant changes in rescue the abnormal expression of genes in STZ induced diabetic rat.

V. CONCLUSIONS

This paper provides insights of the research framework to investigate the differential expression analysis of liver protein in streptozotocin-induced diabetic rat in response to AE treatment. The expected findings from both in vivo and molecular studies may reveal the anti-diabetic properties of the AE and suggest that the extract could be developed as a prospective phytomedicinal plant. The risks involve in this study may include the animals experimental ranging from ethical consideration, pre, during and after experiments being conducted. The physiological status of each of the rat may give different results with different interpretation.

One limitations of the experiment is on the molecular approach. Using four genes to represent the underlying molecular may not be sufficient. Therefore, future works may involve larger number of genes platform system, for example using microarray as a tool to reveal the molecular mechanism of the hypoglycemic of AE. The findings of the expected results from this experiment may provide indications of the changes in gene expression that may reveal underlying mechanism of diabetic pathophysiology, suggesting new potential target for drug discovery.

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