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Hypoglycemic effect of *Octomeles sumatrana* aqueous extract in streptozotocin-induced diabetic rats and its molecular mechanisms

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ABSTRACT

Objective: To investigate the hypoglycemic effect of the aqueous extract of *Octomeles sumatrana (O. sumatrana)* (OS) in streptozotocin-induced diabetic rats (STZ) and its molecular mechanisms. **Methods:** Diabetes was induced by intraperitoneal (*i.p.*) injection of streptozotocin (55 mg/kg) in to male Sprague-Dawley rats. Rats were divided into six different groups; normal control rats were not induced with STZ and served as reference, STZ diabetic control rats were given normal saline. Three groups were treated with OS aqueous extract at 0.2, 0.3 and 0.5 g/kg, orally twice daily continuously for 21 d. The fifth group was treated with glibenclamide (6 mg/kg) in aqueous solution orally continuously for 21 d. After completion of the treatment period, biochemical parameters and expression levels of glucose transporter 2 (Slc2a2), glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PCK1) were determined in liver by quantitative real time PCR. **Results:** Administration of OS at different doses to STZ induced diabetic rats, resulted in significant decrease ($P<0.05$) in blood glucose level in a dose dependent manner by 36%, 48%, and 64% at doses of 0.2, 0.3 and 0.5 g/kg, respectively, in comparison to the STZ control values. Treatment with OS elicited an increase in the expression level of Slc2a2 gene but reduced the expression of G6Pase and PCK1 genes. Morefore, OS treated rats, showed significantly lower levels of serum alanine transaminase (ALT), aspartate aminotransferase (AST) and urea levels compared to STZ untreated rats. The extract at different doses elicited signs of recovery in body weight gain when compared to STZ diabetic controls although food and water consumption were significantly lower in treated groups compared to STZ diabetic control group. **Conclusions:** *O. sumatrana* aqueous extract is beneficial for improvement of hyperglycemia by increasing gene expression of liver Slc2a2 and reducing expression of G6Pase and PCK1 genes in streptozotocin-induced diabetic rats.

1. Introduction

Diabetes mellitus (DM) is a chronic disease that alters the body's metabolism affecting virtually every system in the body. The statistics due to diabetes are alarming and it was estimated by the World Health Organization (WHO) in their 2008 fact sheet on diabetes that more than 180 million people worldwide have diabetes and the number is likely to more than double by 2030 (WHO, 2008). Extensive studies are conducted worldwide to search for treatment of diabetes. Due to the dismal nature of the statistics related to diabetes, a lot of effort is expended to control the disease through the use of oral medications, insulin and other lifestyle changes however, success has been limited. Consequently, the use of traditional plant extracts to treat diabetes and various diseases have been flourished[1]. According to the WHO, more than 150 plants used in the management of diabetes mellitus, thus study of hypoglycaemic plants is encouraged[2]. For example, oral administration of aqueous extract of *Leonotis leonurus* leaf has a beneficial effect in reducing the blood glucose levels as well as lipids[3]. Whereas, it was reported that the leaf and bark extracts of *Butea monosperma* produced antihyperglycemic activity in STZ but the results were insignificant[4]. Traditional plant remedies are empirically used in Asia to treat various diseases and ailments. *Octomeles sumatrana* (*O. sumatrana*) (OS), family Datiscaceae[5] is traditionally used by the

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Kadazandusun people of Sabah, Malaysia as a tonic. The bark is boiled in water and taken orally to treat body ache, muscle sprain, bone pain and stress. Our laboratory has shown this plant to have potent antioxidant capacity similar to vitamins C and E (unpublished data). It is non-toxic as shown *in vitro* and *in vivo*. As a potent antioxidant capacity with safe extract, OS should be explored to establish new pharmacological possibilities for its future development and application. The present study was undertaken to evaluate the potential hypoglycemic activity of the aqueous extract of OS in streptozotocin (STZ)-induced diabetic rats. Effects of OS at different doses were compared to glibenclamide used, a reference hypoglycaemic drug.

An understanding of the many diverse signals that regulate glucose metabolism will aid in the development of pharmacological agents from OS to treat diabetes. Since several anti-diabetic agents, including plant derived drugs target the liver to decrease hepatic glucose production, changes in hepatic expression of the glucose metabolism regulating genes including glucose transporter 2 (Slc2a2), phosphoenolpyruvate carboxykinase (PCK1) and glucose-6-phosphatase (G6Pase) were determined by real time PCR to explore the hypoglycemic mechanism(s) of action of OS aqueous extract.

2. Materials and methods

2.1. Plant material

OS barks were collected from the forest of Tambunan, Sabah, in the north-central region of Malaysian Borneo. Barks were dried, powdered and stored and protected from light at -20 ℃ prior to further use.

2.2. Preparation of the aqueous extract

The extract was obtained as follows; 100 g of dried bark of OS was added in 1 liter of boiling water and boiled for 15 min followed by filtration with filter paper (Milipore filter 0.45 μm Ref HAWP04700) and lyophilization by freeze dryer (Labconco, USA) into powder form. The powder was dissolved in water daily just before administration to experimental rats at the selected doses.

2.3. Animals and treatment

Male Sprague-Dawley rats aged 6-8 weeks (180-250 g) were sued in this study. They were randomly selected and housed under standard environmental conditions (25±1) ℃, 12 h/12 h
light/dark cycle. Animals had free access to water and a standard laboratory diet (carbohydrates: 30%, proteins: 22%, lipids: 12%, vitamins: 3%).

2.4. Induction of experimental diabetes

After an overnight fast, diabetes was induced by intraperitoenal (*i.p.*) injection of a freshly prepared solution of streptozotocin (STZ) (Sigma, St. Louis, Mo) dissolved in 0.1 M cold sodium citrate buffer (pH 4.5) at a dose of 55 mg/kg body weight. Animals were then allowed to drink 5% glucose solution overnight to overcome the drug-induced hyperglycaemia. Control rats were injected with citrate buffer alone. After 1 week, rats with fasting blood glucose levels of greater than 13.8 mmol/L were considered as diabetic and used in the present study[6].

2.5. Experimental design

Rats were divided into six different groups; normal control rats were not induced with STZ and served as reference group. STZ diabetic control rats were given normal saline. Three STZ diabetic groups were treated with OS aqueous extract at 0.2, 0.3 and 0.5 g/kg, twice daily continuously for 21 d. The fifth group received the reference drug, glibenclamide (6 mg/kg) in aqueous solution orally for 21 d.

The doses of the extract were chosen based on dose-response studies that were conducted earlier in our laboratory. Body weight, food and water consumption were measured weekly. Blood was collected from the eye at baseline and during treatment period after 1, and 2 weeks for glucose determination. After 21 d, blood was collected by cardiac puncture for ohaematology and biochemistry tests. At the end of this period the rats were anaesthetized with diethyl ether and killed by cervical dislocation. Liver was excised, rinsed in ice-cold saline and snapped frozen with liquid nitrogen within the period of 2-5 min after death, and stored at -80 ℃ for gene expression studies.

2.6. Parameters

Serum glucose, alanine transaminase (ALT), aspartate aminotransferase (AST) urea and creatinine levels were measured using reagent kits (Instrumentation Laboratory, USA) by ILab Chemistry Analyzer 300 PLUS (Instrumentation Laboratory, USA).

2.7. Gene expression studies

2.7.1. RNA isolation

Total RNA was isolated from fresh frozen liver tissues kept in liquid nitrogen, by TRI Reagent solution according to manufacturer's instructions (Ambion, Austin, Texas, USA). The total RNA concentration was determined by measuring the absorbance at 260 nm. Purity of the extracted RNA was

determined by measuring the ratio of the optical density at 260 nm and 280 nm using a spectrophotometer (BioRad, USA) and ranged between 1.8 and 2.0. The integrity and size distribution of the total RNA was determined using a 1.5 agarose gel. The 18S and 28S RNA bands were visualized under UV light using gel image instrumentation.

2.7.2. Quantitative real-time PCR

Primers specific for Slc2a2, G6Pase, PCK1 and beta-actin genes were designed from the gene sequence of rat (Rattus norvegicus) adopted from the NCBI (National Center for Biotechnology Information) GenBank Database (www.ncbi.nlm.nih.gov) and supplied by NextGene. GenBank accession number code for Slc2a2 is NM_012879.2, for G6pc3 is NM_176077.3, PCK1 is NM_198780.3 and for beta actin gene is NM_031144.2. The sequences of the primers and the size are shown in Table 1. Real-time PCR using SYBR Green chemistry was performed on a RotorGene 6 000 cycler with a 36 well rotor (Corbett Research UK). The run was performed using SensiMix one-step RT-PCR kit with SYBR Green (Quantace, London, UK) according to manufacturer's instructions. Briefly, a reaction volume of 25 μL contained 12.5 μL master mix, 1 μL of 5 μM each forward and reverse primers, and 1 μL of the template RNA at concentration of 100 ng. The reactions were performed under the following conditions: 42 ℃ for 30 min (this step was included to synthesize cDNA), 95 ℃ for 10 min, 40 cycles at 95 ℃ for 15 s, 53 ℃ for 30 min and 72 ℃ for 4 s. Data were acquired on the SYBR channel at the end of each extension step. Melt curves were analysed to check for the absence of mispriming. The possibility of a genomic DNA influence on the results was eliminated by use of primers. Each experiment was performed three times and all samples were run in triplicates. Expression levels for each gene relative to beta-actin were calculated for all samples using the RotorGene software (Version 1.7, Corbett Research) and Microsoft Excel. Analysis of gene expression data was carried out by $^{\Delta\Delta}$CT method of relative quantification[7].

2.8. Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using analysis of variance (ANOVA) followed by Bonferroni post test. Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Serum glucose levels

Serum glucose levels of the experimental period is shown in Figure 1. The diabetic control rats and normal control rats had comparable levels of blood glucose. The STZ control group had significantly ($P<0.01$) higher of serum glucose level compared to normal group. After three weeks of treatment, the diabetic rats that received OS at doses of 0.3 and 0.5 g/kg twice a day and glibenclamide had significantly ($P<0.05$, reduced blood glucose concentrations compared with STZ control group. There was no significant difference in serum glucose levels of STZ treated rats with OS at different doses and STZ treated rats with glybenclamide at the end of the experiment period.

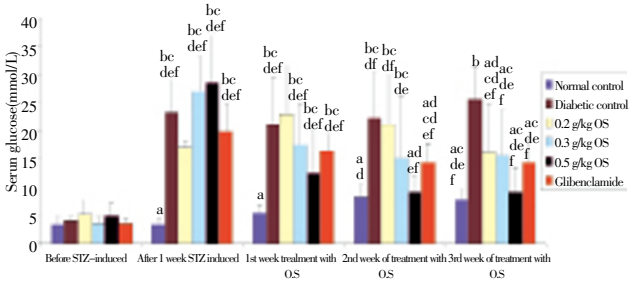

Figure 1. Serum glucose levels.
Different letters within same column indicate significant difference ($P < 0.05$; $n = 6$).

3.2. Body weight, food and water intake

STZ administration caused a significant decrease in the body weight (Figure 2). On the another hand, in STZ treated rats the extract at different doses produced signs of recovery in body weights although not to the level of normal control group. The body weights of the rats given glibenclamide were not significantly different from those diabetic control rats at third week of treatment.

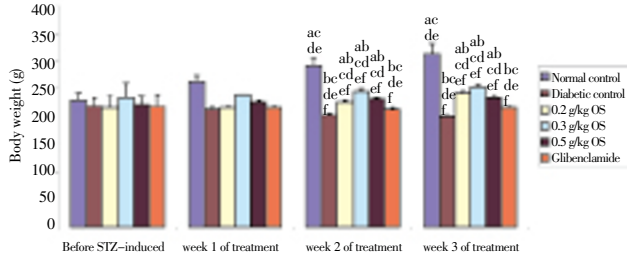

Figure 2. Body weight in experimental animals ($n = 6$).
Different letters within same column indicate significant difference ($P < 0.05$; $n = 6$).

The diabetic control rats consumed significantly more food (Figure 3) and water (Figure 4) compared to the normal group. Treatment of STZ-diabetic rats with OS (0.2-0.5 g/kg) or glibenclamide did not appaerencally effect rats' food or water intake through the three weeks of the treatment. a significant decreasein food and water intake were only seen in diabetic rats that were treated with OS at 0.5 g/kg.

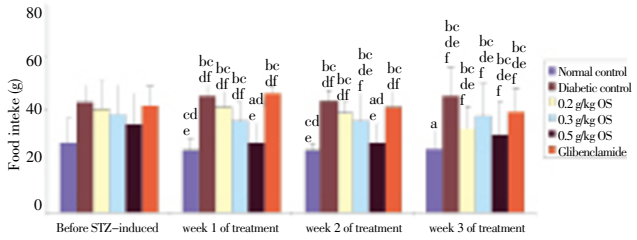

Figure 3. Food intake in experimental animals.
Different letters within same column indicate significant difference ($P < 0.05$; $n = 6$).

2.3. ALT and aspartate AST levels

The STZ control group showed significant elevations in ALT and AST levels compared to normal control group (Table 2). Administration of OS aqueous extract at different doses (0.2, 0.3 & 0.5 g/kg) to STZ diabetic rats caused reversal of the elevations in ALT and AST were elicited by STZ although the values were not lowered to the levels shown by normal non diabetic control group. Creatinine levels were not affected by STZ or by OS or glibenclamide treatment.

Urea levels were significantly higher in STZ diabetic group compared to normal control group. Neither OS nor glibenclamide had much effect on urea levels of diabetic rats.

2.4. Gene expression of glucose metabolism regulating enzymes Slc2a2, PCK1 and G6Pase in the liver

Beta actin gene showed constant expression in different experimental groups and was therefore chosen as the reference gene transcript. A amplification and melting curves analysis were performed which resulted in single product specific melting temperatures as follows: beta actin at 84.3 ℃, Slc2a2 at 82.5 ℃, G6Pase at 86.6 ℃ and PCK1 at 82.3 ℃ (Figure 5a, b). No primer-dimers were generated during the applied 40 real-time PCR amplification cycles. mRNA level of Slc2a2 in STZ diabetic control group were not significantly different from that of normal group (Figure 6). Treatment with OS aqueous extract caused a significant increase in the expression of Slc2a2 mRNA level at doses of 0.3 and 0.5 g/kg by 2 and 2.5 fold, respectively, compared to control diabetic rats. However no significant changes was observed in Slc2a2 mRNA level in treated rats with ether OS at 0.2 g/kg or glibenclamide (Figure 7).

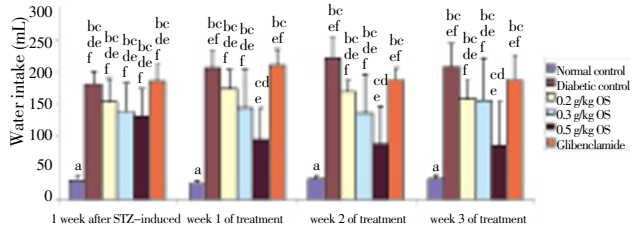

Figure 4. Water intake in experimental animals.
Different letters within same column indicate significant difference ($P < 0.05$; $n = 6$).

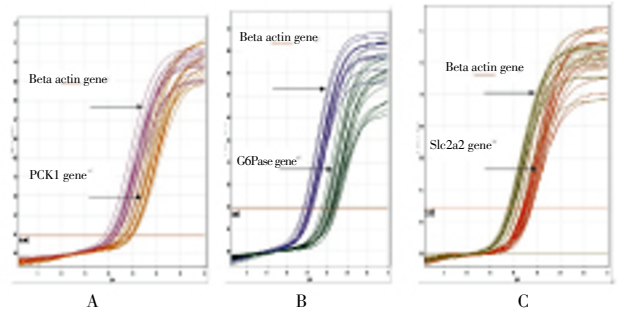

Figure 5. Amplification quality observed by real-time RT-PCR using SYBR green fluorescence history versus cycle number of target genes PCK1 (A), G6Pase (B) and Slc2a2 (C) and reference gene (beta actin) in treated and control samples.

As shown in Figure 8, there was a slightly reduction in the expression of G6Pase in normal rats when compared with the diabetic untreated group. mRNA levels of G6Pase was significantly reduced in OS treated groups at doses of 0.3 and 0.5 g/kg by 53% and 63%, respectively, compared to diabetic untreated group. Whereas, no significant difference was observed in the mRNA levels of G6Pase genes in treated with 0.2 and glibenclamide (Figure 8).

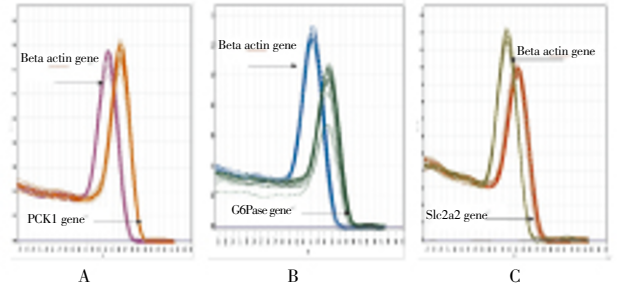

Figure 6. Specific amplification of selected genes.
A melt curve analysis of beta actin, PCK1 (A), G6Pase (B) and Slc2a2 (C) amplification reaction demonstrating the gradual reduction in fluorescence as temperature increases.

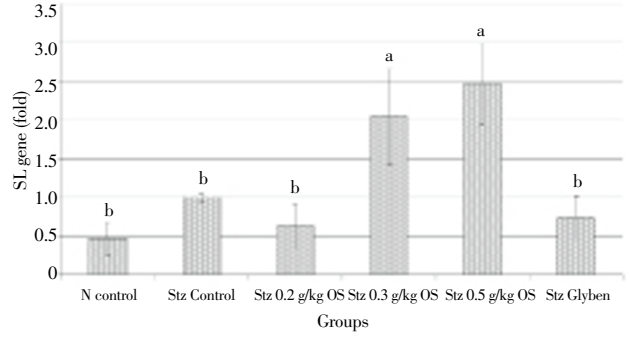

Figure 7. Effect of *O. sumatrana* aqueous extract at different doses on mRNA level of facilitated glucose transporter 2 gene (Slc2a2) in liver of diabetic rats.
Different alphabets indicate significant difference ($P<0.05$) ($n=3$). Run was performed using real time PCR.

As shown in Figure 9, there was a significant reduction ($P<0.05$) in the expression of PCK1 in the groups treated with OS at 0.5 g/kg when compared with the diabetic control

Table 1
Gene name, gene product size, and forward and reverse primer sequences that were used in real time PCR experiment.

Gene name	Fragment size	Forward primer	Reverse primer
NM_031144.2 (Actb)[a]	97	ATGGTGGGTATGGGTCAG	CAATGCCGTGTTCAATGG
NM_012879.2 (Slc2a2)	162	TCTGTGCTGCTTGTGGAG	ACTGACGAAGAGGAAGATGG
NM_176077.3 (G6pc3)	190	ATGATGGCTGAAGACTAC	ACTTGAAGACGAGGTTGAG
NM_198780.3 (Pck1)	171	AACGTTGGCTGGCTCTC	GAACCTGGCGTTGAATGC

[a]Gene used for normalization.

Table 2
AST, ALT, creatinine and urea levels (n = 6).

Groups	AST	ALT	Creatine (mg/dL)	Urea (mg/dL)
xControl	90.0±24.4[a]	54.00±20.10[a]	0.42±0.04[a]	26.00±7.60[a]
stzControl	313.3±86.2[b]	230.20±63.60[b]	0.30±0.08[a]	70.80±13.50[b]
stz0.2 g/kg OS	201.5±68.8[c]	199.00±98.00[a]	0.31±0.10[a]	67.30±14.10[a]
stz0.3 g/kg OS	184.8±41.7[c]	64.80±27.00[a]	0.31±0.10[a]	72.30±36.70[a]
stz0.5 g/kg OS	154.8±63.0[c]	46.50±14.20[a]	0.55±0.09[a]	42.30±27.40[a]
stzGlibenclamide	160.7±53.5[c]	103.20±42.70[a]	0.29±0.10[a]	54.30±26.60[a]

Different letters within same column indicate significant difference (P< 0.05).

group, whereas with 0.2 and 0.3 g/kg treatment statistically insignificant reduction was observed when compared with the diabetic control group.

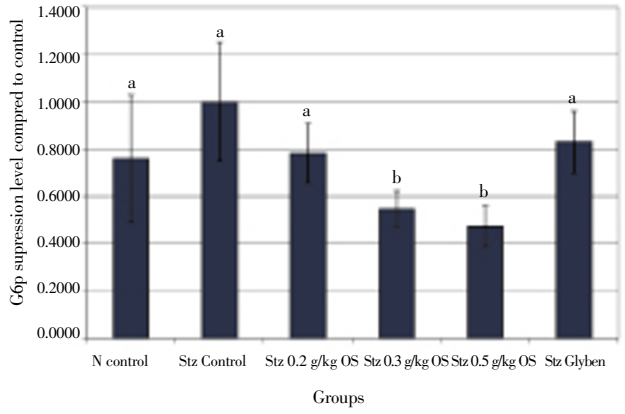

Figure 8. Effect of *O. sumatrana* aqueous extract at different doses on mRNA level of G6Pase in liver of diabetic rats.
Different alphabets indicate significant difference (P<0.05) (n=3). Run was performed using real time PCR.

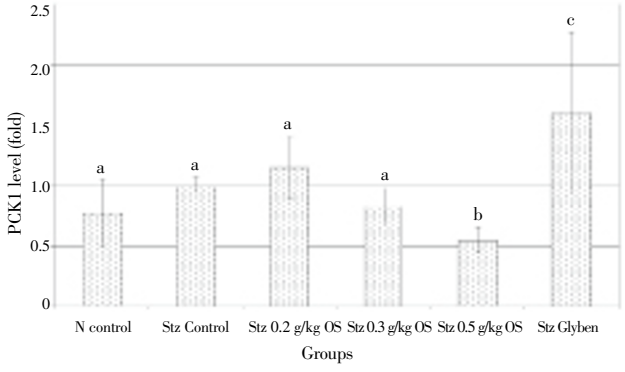

Figure 9. Effect of *O. sumatrana* aqueous extract at different doses on mRNA level of Pck1 in diabetic rat's liver.
Different alphabets indicate significant difference (P<0.05) (n=3). Run was performed using real time PCR.

4. Discussion

Diabetes mellitus (DM) is growing concern and represents one of the main threats to human health. Numerous studies have reported the potential hypoglycemic effect of plant aqueous extract in reducing the glucose level in blood in STZ induced diabetic rats[8-10]. But up to now, there is no a viable study about the hypoglycemic effect of aqueous extract from OS. Therefore, the present study was undertaken to investigate the hypoglycemic effect of aqueous extract of OS in STZ induced diabetic rats. Glibenclamide was used as reference drug to compare the antidiabetic properties of OS. Streptozotocin has been widely used for inducing type I diabetes in a variety of animals by affecting degeneration and necrosis of pancreatic b-cells[11]. Further, the STZ diabetic animals may exhibit most of the diabetic complications, namely, myocardial cardiovascular, gastrointestinal, nervous, vas deferens, liver, kidney, and urinary bladder dysfunctions through oxidative stress[12]. Several workers have reported that STZ-induced diabetes mellitus and insulin deficiency lead to increase blood glucose[13], by inducing necrosis of beta-cells of the islets of langerhans, thus causing hypo-insulin and hyperglycemia[14]. In our study, 3 weeks treatment with aqueous extract of OS twice daily at different doses significantly reduced fasting serum glucose in STZ induced diabetic rat compared to STZ untreated control in a dose dependent manner (P<0.05). The result showed that the serum glucose levels in treated group with 0.3 g/kg and 0.5 g/kg OS were steadily lowered during the whole experiment. Administration of glibenclamide to STZ induced diabetic rats for 21 d, resulted in significant reduction of blood glucose level in comparison to the STZ control group.

The rate-limiting step in the uptake and metabolism of glucose by insulin target cells is glucose transport, which

is mediated by specific glucose transporters of the plasma membrane. Among various glucose transport systems, the liver plays a dual role, as glucose uptake occurs from circulation when gluconeogenesis and glycogenolysis are low; however, glucose is released when gluconeogenesis and glycogenolysis are activated[15]. In the present study, hepatic Slc2a2 expression was higher in diabetic STZ animals compared to normal rats. Friedman *et al*[16], have shown that the expression of hepatic Glut2 was increased in diabetic animals compared to normal rats. In contrast to the hepatic Glut2, the liver Slc2a2 expression was significantly higher in the treated groups with SO at 3 and 0.5 g/kg when compared with the control group.

The treatment significantly reduced the expression of the gene encoding the regulatory enzyme of gluconeogenesis and glycogenolysis (G6Pase and PCK1) in the liver of STZ rats. These results indicate that OS extract affect the last step in gluconeogenesis and glycogenolysis. Glucose is formed from gluconeogenic precursors in liver, and also from glycogen in liver. Both gluconeogenesis and glycogenolysis result in the formation of glucose 6-phosphate (Glc-6-P), which has to be hydrolysed by G6Pase before being released as glucose into the circulation. G6Pase thus plays a critical role in blood glucose homoeostasis.

The present study reveals that aqueous extract of OS, represses hepatic PCK1 and G6Pase gene expression. Because of insulin level was not measured in the present study, so we were not able to address weather if any stimulation of insulin secretion is likely to have taken place after administration of the extract. Subsequently, some hypothetical suggestions can be made, that the plasma glucose lowering effect in the absence of plasma insulin concentration suggests that OS treatment may involve in an insulin independent mechanism. It can be postulated to involve an antioxidant mechanism as study from our laboratory has shown that OS aqueous extract was shown to be a potent antioxidant (not published data).

Glucose transporter 2 (Slc2a2), is present in the liver under both gluconeogenic and glycolytic conditions. Under the former condition, Slc2a2 activity would be essential for both glucose secretion and keeping the intracellular Glu-6-P concentration low and thus avoid permanent activation of glycolytic and lipogenic genes[17]. In the present study, thus, increase the expression of hepatic GLUT2 might add another efficient mechanism through response to insulin action. Two groups[18], reported in murine hepatocytes that GLUT2 and the insulin receptor (IR) seemed to colocalize and cointernalize in the endosomal fraction in response to insulin action. Such IR-GLUT2 complexes were increased in a mouse model of improved hepatic insulin sensitivity[19].

STZ inductions caused a significant reduction in the body weight gain of the rats (diabetic control group) and were significantly lower than non-diabetic control rats. Polyuria, characteristic symptom of diabetes that could be a factor for the lowered body weights of STZ-diabetic rats compared to control rats[20]. Other studies showed that plant extracts may by themselves contribute to body weight loss in animals[21]. However, oral administration of OS aqueous extract to STZ-diabetic rats did not much change the body weights of the STZ-diabetic rats. Diabetic rats treated with *Syzygium cordatum* leaf extract did not cause any significant differences in body weight compared to the untreated rats[22]. In our study, STZ induced diabetic rats were shown a significant higher level of the average food and water consumption than the normal rats and than that in the STZ treated rats groups, which seems to be due to STZ. Our findings are in agreement with the previous findings reported[23]. Serum ALT and AST levels were determined to evaluate the hepatic functions in hyperglycemia rats. Measurement of enzymatic activities of aminotransferase (AST and ALT) is of clinical and toxicological importance, as changes in their activities are indicative of tissue damage by toxicants or in disease conditions[24]. In normal rats, the normal level of serum AST, ALT in treated and untreated rats suggests that the extract may not be toxic at least to the liver at the concentration employed. However, the results of our study showed that STZ diabetes in rats caused significant increases in the levels of AST and ALT in diabetic rats, these findings were also reported by many other workers[25]. Voss *et al*[26], proposed that STZ in hyperglycemic animals caused a time dependent rise in AST and ALT levels. Elevated levels of these transaminases could be responsible for the increased gluconeogenesis and ketogenesis that are observed in diabetes[27,28]. Therefore, treatment with OS at different doses caused significant reduction of AST and ALT compared to untreated STZ diabetes rats in a dose dependent manner, that`s suggested that the OS prevented liver damage further studies should carried out to investagate this activity and the mechanism of action. The diabetic hyperglycemia induces elevation of the serum levels of urea which were considered as significant markers of renal dysfunction[29]. The result show there was significant increase in the level of serum urea in the diabetic control groups compare with the non diabetic control groups.

In conclusion, the aqueous extract of OS was found to be effective for improving the hyperglycemia in STZ induced diabetic rats by reducing the expression of G6Pase and PCK1 genes while increasing the expression of Slc2a2 gene in the liver tissue.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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