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INSULIN-MIMETIC ACTIVITY OF STEVIOSIDE ON DIABETIC RATS: BIOCHEMICAL, MOLECULAR AND HISTOPATHOLOGICAL STUDY

Osama M. Saleh[1,2,*], Nabil S. Awad[3], Mohamed M. Soliman[4,5], Ahmed A. Mansour [1,6], Mohammed A. Nassan[4,7]

[1]Medical Biotechnology Department, Faculty of Applied Medical Sciences (Turabah), Taif University, KSA. [2]National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Nasr City, Cairo, Egypt. [3] Department of Genetics, Faculty of Agriculture and Natural Resources, Aswan University, Aswan, Egypt. [4]Medical Laboratories Department, Faculty of Applied Medical Sciences (Turabah), Taif University, KSA. [5]Biochemistry Department, Faculty of Veterinary Medicine, Benha University, Benha, Egypt. [6]Genetics Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. [7] Pathology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.
***Corresponding Author E-mail:** omoseilhy@hotmail.com

Abstract

Background: Stevioside has been used as a medication for reducing glucose levels in diabetic patients. The exact mode of action is still unclear. Therefore, the current study outlined the molecular and biological roles of stevioside in treatment of diabetes.
Materials and Methods: Diabetic male Wistar rats were treated with stevioside and metformin as a medication for diabetes rats for 4 weeks. Biochemical, molecular and histopathological studies have been done to evaluate the therapeutic effect of stevioside on minimizing levels of glucose and its related gene expression in diabetic rats.
Results: Stevioside administration normalized kidney and liver biomarkers, restored alterations in antioxidants activity and lipid profiles. Moreover, stevioside increased insulin and leptin secretion that are decreased in diabetic rats to the normal levels. For mRNA expression, stevioside up-regulated the expressions of PK and IRS-1 genes, which are down-regulated in diabetic rats, and was very effective in the down-regulation of CPT-1 mRNA expression. At the cellular levels; stevioside normalized the histopathological changes induced in pancreas.
Conclusion: Stevioside has insulin like effects and is useful for diabetic patient's therapy.

Key words: *Stevioside; Molecular effects; Insulin like activity, Diabetes.*

Abbreviations: ANOVA: analysis of variance; ATP: adenosine Tri Phosphate; BW: Body Weight; cDNA: Complementary Deoxy Ribonucleic Acid; CNT: Control Treatment; CPT-1: CarnitylPalmityl Transferase-1; D: Diabetic Group; DAB: Diaminobenzidine; DEPC: Diethylpyrocarbonate; DNA: Deoxy Ribonucleic Acid; G3PDH: Glyceraldehyde-3-Phosphate Dehydrogenase; GOT: Glutamate Oxalacetate Transaminase; GPT: Glutamate Pyruvate Transaminase; GR: Glutathione Reductase; H and E: Hematoxylin and Eosin; HDL: High Density Lipoproteins; HRP: Horseradish Peroxidase; IRS-1: Insulin Receptor Substrate-1; MDA: Malondialdehyde; MET: Metformin Treatment; NBF: Neutral Buffered Formalin; OD: Optical Density; PBS: Phosphate Buffered Saline; PK: Pyruvate Kinase; PM: Pico Mol; ROS: Reactive Oxygen Species; RT-PCR: Reverse-Transcribed Polymerase Chain reaction;SEM: Standard Error of Means; SOD: Superoxide Dismutase; ST: Stevioside treatment; STZ: Streptozotocin; TBE: Tris-Borate-EDTA; TC: Total Cholesterol; TG: Triglycerides; UV: Ultra Violet.

Introduction

Diabetes Mellitus is one of the most commonly occurring problems around the globe (Hossain et al 2011). Defects in insulin secretion or low sensitivity of the tissue to insulin lead to hyperglycemia, hypercholesterolemia, and hypertriglyceridemia which cause diabetes (Mishra 2009). Uncontrolled glucose level in the blood is due to the disturbance of insulin level (Dasgupta 2004). Diabetes Mellitus is expected to reach 300 million people in 2025 especially type II which associated with more calories nutrition and obesity (Zimmet et al 2001, 2003).Treatment of diabetes depends mainly on reduction of hyperglycemia using different substances such as; biguanides, thiazolidinediones, sulfonylureas D-phenylalanine and α-glucosidase inhibitors in addition to insulin. Debatable efficiency and some harmful side effects of these drugs urged patients to search about new compounds especially from natural herbs to treat diabetes (Moller 2001). Herbal medication is a considered less toxic and with little side effects compared to synthetic drugs (Bailey and Day 1989). Oxidative stress is associated with diabetes mellitus incidence, so using some natural products such as stevioside can play a crucial role as antioxidant and ameliorate diabetes biohazards (Sharma et al 2012).

Stevia shrub (*Stevia rebaudiana* Bertoni) is known as high quality bio-sweeter calorie free (Bharathi 2003; Preethi 2011). Leaves of stevia shrub are very effective to protect rats against streptozotocin (STZ) induced diabetes, reduces the risk of oxidative stress produced as a result of hyperglycemia (Badawi et al 2005), and ameliorates liver and kidney damage (Naveen et al 2013). Stevioside is the major component of stevia plants and is sweeter than sucrose (Maier et al 2003) with no side effects (Megaji et al 2005). Stevioside has an ability to decrease the bad effect of hyperglycemia probably through enhancing insulin secretion and utilization in diabetic rats (Jeppesen et al 2003; Gregersen et al 2004; Chen et al 2005; Seema, 2010). Many searches reported that stevioside has been used for treatment of various diseases such as cancer (Yasukawa et al 2002), diabetes (Lailerd et al 2004), obesity, cavities, hypertension (Dyrskog et al 2005), fatigue, depression, and in cosmetic and dental preparations (Duke 2006).

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Stevioside extract can be used as anti-fungal, anti-bacterial, anti-viral and anti-inflammatory agent (Lee et al 2001). Moreover, the extract of *Stevioside rebaudiana* has potential hepato-protective activity (Das and Kathiriya 2012). Stevioside has been found to increase insulin sensitivity in rodent models (Chang et al 2005) and to have beneficial effects on blood glucose and insulin levels in human studies which suggest it may have a role in food intake regulation (Gregersen et al 2004). Stevioside extract helps in reduction of postprandial blood glucose and insulin levels due to assistance of glucose regulation (Anton et al 2010). Stevioside has been used to decrease body weight (Dutta et al 2010). As known, diabetes incidence is accompanied by disturbance in carbohydrate metabolism, changes in lipid profiles, oxidative stress and changes in insulin and glucose levels. Therefore, the aim of the current study is to examine the beneficial effects of stevioside on STZ diabetic rats at the biochemical, molecular and immune-histochemical levels.

Methods

Chemicals and Kits

Streptozotocin (STZ), ethidium bromide and agarose were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Wistar albino rats were purchased from King Fahd center for Scientific Research, King Abdel-Aziz University, Jeddah, Saudi Arabia. Serologic kits for glutamate pyruvate transaminase (GPT), glutamate oxalacetate transaminase (GOT), catalase, malondialdehyde (MDA), glutathione reductase, superoxide dismutase (SOD), total cholesterol, triglycerides (TG), high density lipoproteins (HDL), creatinine and urea were purchased from Bio-diagnostic Co., Dokki, Giza, Egypt. The deoxyribonucleic acid (DNA), 100 bp ladder was purchased from MBI, Fermentas, Thermo Fisher Scientific, USA. Qiazol for RNA extraction and oligo dT primers were purchased from QIAGEN (Valencia, CA, USA). Anti- rat insulin primary antibody and rat ABC staining system were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals, Experimental Design and Sampling

All animal procedures were approved by the Ethical Committee Office of the dean of scientific affairs of Taif University, Saudi Arabia. Fifty male Sprague Dawley rats, 3 months old, weighing 200–280 g were used for this study. For acclimatization, animals were handled daily and kept under observation for one week before the onset of the experiment. The animals were kept at 12-h light-dark cycle and gained access to food and water ad libitum. Twenty rats were handled and divided into 2 groups control (CNT) without any treatment and control plus stevioside (CNT + ST) received stevioside 300 mg/kg B.W based on study of (Singh et al., 2013) for 4 weeks. Remaining thirty rats were injected intraperitoneally by STZ in a dose of 60 mg/kg B.W in citrate buffer after overnight fasting (Soliman et al 2012). To avoid sudden death due to hypoglycemia, rats supplemented with 5% glucose solution for the next 12 hrs. Tail vein glucose level was measured using commercial available glucose detector set after 72 hrs after STZ injection. Rats with glucose levels over 200 mg/dL considered diabetic. Diabetic rats were randomly divided into 3 groups as below:
Diabetic group (D) served as positive control and received water orally. Diabetic plus metformin group (D + MET) received metformin (300 mg /kg B.W orally) dissolved in water for 4 weeks (Ismail et al 2015). Diabetic plus stevioside (D +ST) received stevioside for 4 weeks. Twenty four hours after last medications, all rats were sacrificed after anesthetization by diethyl ether inhalation. Blood and tissues were collected from slaughtered rats. Serum was extracted after blood centrifugation for 10 min at 4000 xg. For gene expression, liver tissues were kept in Qiazol® reagent (Life Technologies, USA) at -80 ˚C in deep freezer for ribonucleic acid (RNA) extraction and pancreas tissues were placed in 10% neutral buffered formalin (NBF) at room temperature for 24 hours for histopathological and immunohistochemical study.

Serum Chemistry Assays

Triglycerides (TG), total cholesterol (TC), and high-density lipoproteins (HDL) were measured using commercial spectrophotometric analysis kits (Bio-Diagnostic Company, Giza, Egypt). Insulin and leptin levels were measured using spectrophotometric commercial kits bought from Chrystal Chem. Co, Downers Grove, IL 6051, USA. Glucose was measured using commercial glucose monitoring set (ACCU-Chek).

RNA Extraction, Complementary Deoxyribonucleic Acid (Cdna) Synthesis and Semi-Quantitative PCR Analysis

Total RNA was extracted from liver tissue samples as previously discussed (Bancroft et al 2008). RNA concentration and purity were determined spectrophotometrically after measuring OD at 260 and 280 nm. The RNA integrity was confirmed after running in 1.5% denaturated agarose gel stained with ethidium bromide. The ratio of the 260/280 optical density of all RNA samples was 1.7-1.9.A mixture of 3 µg total RNA and 0.5 ng oligo dT primer (Qiagen Valencia, CA, USA) were used for cDNA synthesis in a total volume of 11 µl sterilized DEPC water was incubated in the Bio-Rad T100™ thermal cycler at 65°C for 10 min for denaturation. Then, 2 µl of 10X RT-buffer, 2 µl of 10 mM dNTPs and 100 U Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (SibEnzyme. Ak, Novosibirsk, Russia) were added and the total volume was completed up to 20 µl by DEPC water. The mixture was then re-incubated in BIO-RAD thermal cycler at 37 °C for one hour, then at 90 °C for 10 min to inactivate the enzyme. For semi-quantitative RT-PCR analysis, specific primers for examined genes (Table 1) were designed using Oligo-4 computer program and synthesized by Macrogen (Macrogen Company, GAsa-dong, Geumcheon-gu. Korea). PCR was conducted in a final volume of 25 µl consisting of 1 µl cDNA, 1 µl of 10 pM of each primer (forward and reverse), and 12.5 µl PCR master mix (Promega Corporation, Madison, WI, USA), the volume was brought up to 25 µl using sterilized, deionized water. PCR was carried out using Bio-Rad T100™ thermal cycler machine with the cycle sequence at 94°C for 5 min one cycle, followed by 31 cycles (Table 1) each of which consists of denaturation at 94 °C for one min, annealing at the specific temperature corresponding to each primer (Table 1) and extension at 72 °C for one min with an additional final extension at 72 °C for 7 min. As a reference, expression

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of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was examined (Table 1). PCR products were visualized under UV light after electrophoresis on 1.5% agarose (Bio Basic, Markham, ON, Canada) gel stained with ethidium bromide in TBE (Tris-Borate-EDTA) buffer. PCR products were photographed using gel documentation system. The intensities of the bands were quantified densitometrically using Image J software version 1.47 (http://imagej.en.softonic.com/).

Table (1): PCR conditions and primers sequence of examined genes.

Gene	Product size (bp)	Annealing	Direction	Sequence (5'-3')
PK	229	52	Sense	ATTGCTGTGACTGGATCTGC
			Antisense	CCCGCATGATGTTGGTATAG
IRS-1	337	53.5	Sense	GCCAATCTTCATCCAGTTGC
			Antisense	CATCGTGAAGAAGGCATAGG
CPT-1	628	52	Sense	TATGTGAGGATGCTGCTTCC
			Antisense	CTCGGAGAGCTAAGCTTGTC
GAPDH	309	52	Sense	AGATCCACAACGGATACATT
			Antisense	TCCCTCAAGATTGTCAGCAA

Pancreatic Histolopathology and Immunohistochemistry

Pancreatic tissues were removed from the rats and fixed over night in 10% buffered neutral formalin solution. Fixed tissues were processed routinely including washing, dehydration, clearing, paraffin embedding, casting, sectioning to 5 µm sections for using in hematoxylin and eosin staining (Soliman et al 2014). For immunohistochemistry, tissue sections of pancreas were deparaffinized then treated with 3% H_2O_2 for 10 min to inactivate peroxidases, heated in 10 mM citrate buffer at 121°C for 30 min for antigen retrieval, blocked in 5% normal serum for 20 min, and incubated with a primary polyclonal anti-insulin (dilution, 1:100 in PBS; sc-9168, Santa Cruz Biotechnology) antibody overnight at 4°C. After three extensive washes with PBS, sections were incubated with a biotin-conjugated secondary antibody (dilution, 1:2000 in PBS; sc-2040, Santa Cruz Biotechnology) for 20 min at 32°C. After further incubation with horseradish peroxidase (HRP)-labeled streptavidin, antibody binding was visualized with diaminobenzidine and sections were counterstained with hematoxylin. Tissue slides were visualized using Wolfe S9-0982 microscope and figures were captured using Moticam digital camera.

Statistical Analysis

Results are represented as means ± standard error of means (SEM). Data were analyzed using analysis of variance (ANOVA) and *post hoc* descriptive tests by SPSS software version 11.5 for Windows (SPSS, IBM, Chicago, IL, USA) with $p<0.05$ regarded as statistically significant. Regression analysis was performed using the same software.

Results and Discussion

Effect of Stevioside on Serum Glucose, Insulin and Leptin Levels in Diabetic Rats

Treatment of diabetic rats with stevioside decreased the increase in glucose levels reported in diabetic rats that is coincided with the decrease seen in metformin administered diabetic rats. The changes in glucose levels is due to the increase in insulin secretion reported in stevioside administered diabetic rats as seen in (Table 2). Leptin was decreased in diabetic rats and administration of metformin and stevioside normalized and increased leptin levels in a way to increase peripheral glucose utilization and to control serum glucose levels (Table 2).

Table (2): Serum changes in glucose levels, insulin and leptin in diabetic rats and after stevioside administration for 2 months.

	Glucose (mg/dL)	Insulin (µIU/ml)	Leptin (ng/ml)
Control	83.0 ± 9.7	35.3 ± 2.3	12.6 ± 3.1
Diabetes	462.3 ± 37.8*	8.3 ± 0.5*	9.1 ± 5.1*
Diabetes + Metformin	120.0 ± 10.1#	27.2 ± 3.4#	17.2 ± 5.3#
Control + Stevia	96.7 ± 8.1	31.3 ± 2.3	10.7 ± 1.3
Diabetes + Stevia	85.7 ± 11.1#$	45 ± 3.1#	19.4 ± #

Values are means ± standard error (SEM) for 3 independent experiments per each treatment. Values are statistically significant at *$p<0.05$ Vs. control; #$p<0.05$ Vs. diabetic group #$p<0.05$ Vs. diabetic metformin group.

Effect of Stevioside on Kidney and Liver Biomarkers in Diabetic Rats

As seen in (Table 3), diabetes significantly increase serum levels of urea, creatinine, GPT and GOT. Administration of stevioside to diabetic rats normalized such increase in kidney and liver biomarkers (Table 3). The results reported for stevioside in diabetic rats are the same reported for control positive metformin administered rats.

Effect of Stevioside on Oxidative Stress and Antioxidants Levels in Diabetic Rats

Changes in malondialdehyde (MDA) levels are listed in Table 4. Hyperglycemia causes high ROS production and, in turn, leads to high MDA levels in serum (7 fold increase). MDA was increased in diabetes while, stevioside administration decreased the increase in MDA levels (Table 4). In parallel, antioxidants levels represented by glutathione reductase (GR), catalase and superoxide

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dismutase (SOD) were decreased in diabetic rats. STZ administration resulted in significant reduction of antioxidant enzymes SOD and catalase by 60% compared to control group. When rats were administered with stevioside; the enzyme activity was restored to normal and was significant when compared to diabetic group (Table 4). Stevioside also increased the level of antioxidant enzymes and could be effective through scavenging these free radicals.

Table (3): Serum changes in kidney and liver function parameters in diabetic rats and after stevioside administration for 2 months.

	Urea (mg/dL)	Creatinine (mg/dL)	GPT (U/l)	GOT (U/l)
Control	43.0 ± 9.2	0.8 ± 0.1	103.6 ± 7.7	90.6 ± 7.6
Diabetes	111.7 ± 14.8*	2.4 ± 0.2*	169.1 ± 15.1*	181.1 ± 5.1*
Diabetes + Metformin	69.0 ± 22.4#	1.2 ± 0.4#	99.2 ± 25.3#	121.7 ± 14.9#
Control + Stevia	41.7 ± 11.4	1.3 ± 0.1	87.7 ± 4.3	97.7 ± 8.9
Diabetes + Stevia	43.7 ± 4.1#$	0.7 ± 0.2#	101.7 ± 5.2#	83.7 ± 12.9#$

Values are means ± standard error (SEM) for 3 independent experiments per each treatment. Values are statistically significant at *p<0.05 Vs. control; #p<0.05 Vs. diabetic group #p<0.05 Vs. diabetic metformin group.

Table (4): serum changes in malondialdehyde (MDA) and antioxidants levels in diabetic rats and after stevioside administration for 2 months.

	MDA(nmol/g tissue)	Catalase (U/g tissue)	GR (U/g protein)	SOD (U/g protein)
Control	4.5 ± 0.1	31.4 ± 2	9.4 ± 0.4	11.5 ± 0.3
Diabetes	28.5 ± 1.8*	8.7 ± 0.5*	3.1 ± 0.1*	2.9 ± 0.2*
Diabetes + Metformin	14.1 ± 3.8#	26.4 ± 2.4#	7.5 ± 1.0#	9.8 ± 1.2#
Control + Stevia	8.5 ± 2.0	30.1 ± 0.1	8.8 ± 0.1	12 ± 0.2
Diabetes + Stevia	12.1 ± 1.2#	38.7 ± 1.3#$	8.8 ± 0.4#	13.1 ± 0.4#

Values are means ± standard error (SEM) for 3 independent experiments per each treatment. Values are statistically significant at *p<0.05 Vs. control; #p<0.05 Vs. diabetic group #p<0.05 Vs. diabetic metformin group.

Effect of Stevioside on Lipid Profiles in Diabetic Rats

Table (5) shows that incidence of diabetes in rats induced significant increase in total cholesterol and triglyceridemia (dyslipidemia) and a decrease in levels of high density lipoproteins (HDL). Stevioside administration normalized the increase in total cholesterol and TG levels. Moreover, it increased HDL levels relative to diabetic rats. The effects induced by stevioside are relatively the same reported for metformin administered rats (Table 5).

Table (5): Serum changes in lipid parameters in diabetic rats and after stevioside administration for 2 months.

	Cholesterol (mg/dl)	TG (mg/dl)	HDL (mg/dl)
Control	9 3.7 ± 10.2	53.3 ± 2.8	19.7 ± 0.9
Diabetes	252.7 ± 18.2*	128.3 ± 11.8*	8.9 ± 4.1*
Diabetes + Metformin	161.2 ± 21.8#	85.7 ± 9.1#	16.2 ± 0.6#
Control + Stevia	98.3 ± 9.2	65 ± 10.7	18.7. ± 0.9
Diabetes + Stevia	88.7 ± 9.2#	69 ± 9#	19 ± 1.5#

Values are means ± standard error (SEM) for 3 independent experiments per each treatment. Values are statistically significant at *p<0.05 Vs. control; #p<0.05 Vs. diabetic group #p<0.05 Vs. diabetic metformin group.

Effect of Stevioside on Pyruvate Kinase (PK), Insulin Receptor Substrate-1 (IRS-1) and Carnitylpalmityl Transferase-1 (CPT-1) Genes Expression in Diabetic Rats

The effect of stevioside on the expression of some genes related to carbohydrate and lipid metabolism ameliorated during diabetes incidence in rats have been tested. As shown in Figure (1), expression of PK was down-regulated in diabetic rats and increased in metformin administered diabetic rats. Stevioside administration induced an increase in PK mRNA expression same reported for metformin administered rats (Figure.1[1st] panel). Similar changes in mRNA expression of IRS-1 was occurred and was the same like PK (Figure. 1[2nd] panel), PK is responsible for the increase in hepatic glycolysis with up-regulation in insulin receptor expression to help in peripheral glucose utilization. The expression of CPT-1 was increased during diabetes because of lipolysis. Metformin administration failed to inhibit the up-regulation occurred during diabetes (Figure. 1 [3rd] panel). Interestingly, stevioside

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down-regulated the increase in mRNA expression of CPT-1 reported in diabetic and metformin administered rats that mean it inhibited lipolysis associated with diabetes.

Figure 1:

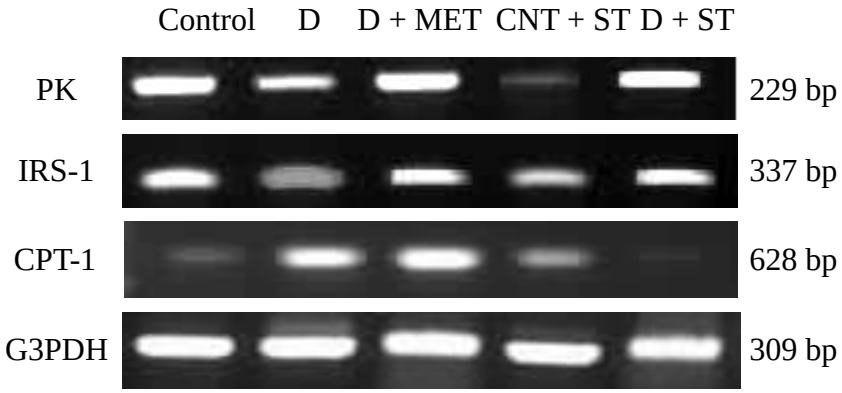

Figure1: Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of PK, IRS-1 and CPT-1 expression in the liver tissue of the control, diabetes, metformin-administered rats and stevioside administered rats. RNA (1 µg) was extracted, reverse transcribed and RT-PCR analysis was conducted to assess PK, IRS-1 and CPT-1 expression, as described in the materials and methods.

Effect of Stevioside on Pancreas Histology and Immunohistochemistry

Control group showed normal pancreatic tissue with normal Islet's of Langerhans (IL) forming cords separated by blood capillaries together with normal pancreatic acini with its basal basophilia and apical acidophilia (Figure 2A).Pancreatic tissues of STZ administered group showed atrophy of pancreatic Islet's with infiltration of inflammatory cells and deposition of amyloid like materials (Figure 2B). Pancreatic tissues of diabetic group treated with stevioside showed restoration of normal appearance of pancreatic Islet's and pancreatic acini (Figure 2C). Insulin immune-stained rat pancreatic tissues of control group showed normal expression of insulin within pancreatic Islet's (Figure 2D) while that of STZ administered group showed mild expression of insulin within pancreatic Islet's (Figure 2E). Pancreatic tissues of diabetic group treated with stevioside showed increase in the immune expression of insulin within pancreatic Islet's (Figure 2F). Diabetes is a chronic metabolic disorder affects approximately 3% of population worldwide (Kim et al 2006). As known, sustained reductions in hyperglycemia will decrease the risk of developing micro-vascular diseases and reduce diabetes complications (Gaster and Hirsch 1998). Usage of synthetic oral hypoglycemic drugs to control diabetes has several adverse effects and high rates of secondary failure (Kim et al 2006). Those adverse effects made diabetic patients to think to use herbal medication that has similar degree of efficiency without side effects. We have shown in this study that administration of stevioside for 4 weeks in STZ diabetic rats induced insulin mimetic effects. The results showed that insulin deficiency is strongly associated with metabolic dyslipidemia in diabetes. Moreover, our findings have confirmed the anti-diabetic activity of stevioside in STZ diabetic rats. Stevioside administration not only reduced blood glucose levels for diabetic rats, but also normalized and restored insulin levels in diabetic rats compared to initial insulin control values. Parallel findings were reported by various studies for another herbal medication; as 45 days treatment of cinnamaldehyde increases serum insulin levels in STZ induced diabetic rats (Anand et al 2010; SubashBabu et al 2007; Soliman et al 2013). This findings was explained that the over insulin secretion from still working β-cells in a way to overcome hyperglycemia of diabetic rats (Kim et al 2006**)**. New findings were reported here is the ability of stevioside to normalize the decrease in leptin levels reported in diabetic patients. Leptin is a protein mainly secreted from white adipose tissue to increase peripheral glucose utilization and energy expenditure (Soliman et al 2006). Therefore, stevioside possess the ability to control diabetes through regulation of both insulin and leptin secretion from still working β- cells of pancreas and white adipose tissue respectively.

STZ causes diabetes by the rapid depletion of β-cells, which leads to a reduction in the insulin release. An insufficient release of insulin causes hyperglycemia, which results in oxidative damage by the generation of reactive oxygen species (Kangralkar et al 2010) and the development of diabetic complications. The results of this study showed increased serum insulin level in the diabetic stevioside administered rats. This means, stevioside would enhance the number of β-cells of pancreatic Islets in diabetic treated rats (Shivanna et al 2013). Moreover, hyperglycemia increases the generation of free radicals by glucose auto-oxidation and the increment of free radicals may lead to liver cells damage. The increase in oxygen free radicals in diabetes could be primarily due to the increase in blood glucose levels and secondarily due to the effects of the diabetogenic agent STZ (Shukla et al 2011). In this study, stevioside showed strong free radical scavenging and antioxidant activities and also provoked a protective effect on DNA damage caused by hydroxyl radicals (Shukla et al 2009). Here, stevioside prevented the damage occurred in diabetic rats not only at kidney and liver biomarkers, but also at the antioxidants secretion levels. However, the mechanism of action of stevioside as a herbal drug in reducing the diabetes is not known. In fact, it has been found that herbal medications used in human diabetes also have a significant antioxidant activity and that confirmed in our study. Therefore, it may be possible that stevioside may reduce the effect of inflammatory cytokine release during diabetes which may be one of the causative agents for the tissue distraction and insulin resistance (Saghizadeh et al 1996**)**. GPT and GOT are the common intracellular enzymes that increased due to the liver damage induced by diabetes (Can et al 2004). In this study, we clearly showed that the high levels of GPT and GOT in serum of the STZ group. In contrast, they were decreased in the stevioside administered rats and the same was reported to the changes in urea and creatinine. Stevioside administration caused a significant decrease in the levels of GPT, GOT, urea and creatinine levels and had a

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protective effect on the kidney and liver damage of diabetic treated rats as reported in this study and others (Abdelsattarelbatran et al 2006). SOD, catalase and GR are considered primary antioxidant enzymes, and are involved in direct elimination of reactive oxygen species (Bhor et al 2004). In our study, administration of stevioside increased SOD, catalase and GR enzymes to reverse the oxidative damage induced by STZ.To understand the molecular mechanism of stevioside actions, we examined the mRNA expression of genes involved in lipid and glucose metabolism in liver of diabetic rats. STZ administration induced dyslipidemia, while, stevioside normalized the increase in cholesterol and TG and the decrease in HDL with changes in CPT-1 mRNA expression. Similar findings were reported by (Soliman et al 2012) for cinnamon extract useful medication for diabetes treatment. HDL-cholesterol is good for health, since it facilitates mobilization of triglycerides and cholesterol from the plasma to the liver (Miller et al 1985). As known, glycogen is the storable form of glucose inside cells especially in liver and skeletal muscles and its accumulation indicates direct reflection of insulin activity. STZ causes destruction of β-cells of pancreas resulting in marked decrease in insulin levels, it could be predicted that glycogen levels in tissues (muscle and liver) decrease as the influx of glucose in liver is inhibited in the absence of insulin and recovers on insulin treatment (Vats et al 2004). Our findings showed that administration of stevioside to diabetic rats induced significant increase in PK mRNA expression and were coincided with the increase in the expression of IRS-1. That means; in diabetic rats, stevioside improved PK enzyme expression levels which contribute to glucose homeostasis. The PK activity decreases as the result of diabetes and increases by the administration of insulin to diabetic rats in the liver tissues (Yamada and Noguchi 1999). The altered activity during diabetic conditions could be expected to diminish the metabolism of glucose and ATP production. The decrease in activity of PK in the liver tissue of STZ diabetic rats is the cause of glycolysis reduction (Anand et al 2010). Moreover, it has been shown that enhanced fatty acids β-oxidation in 3T3-L1 cells is associated with a concomitant increase in the expression of CPT-1, a key enzyme in transferring acetyl-CoA into mitochondria for β-oxidation (Ejaz et al 2009). Therefore, in this study stevioside in diabetic rats decreased CPT-1 expression which due the body to increase glycolysis represented by the increase in PK expression and consequently control lipolysis and increased hepatic glucose utilization. At the cellular levels, STZ showed atrophy in pancreatic β-cells and absence in insulin immune-staining. Stevioside administration restored and showed normal pancreatic cells and increase in insulin immune-staining.

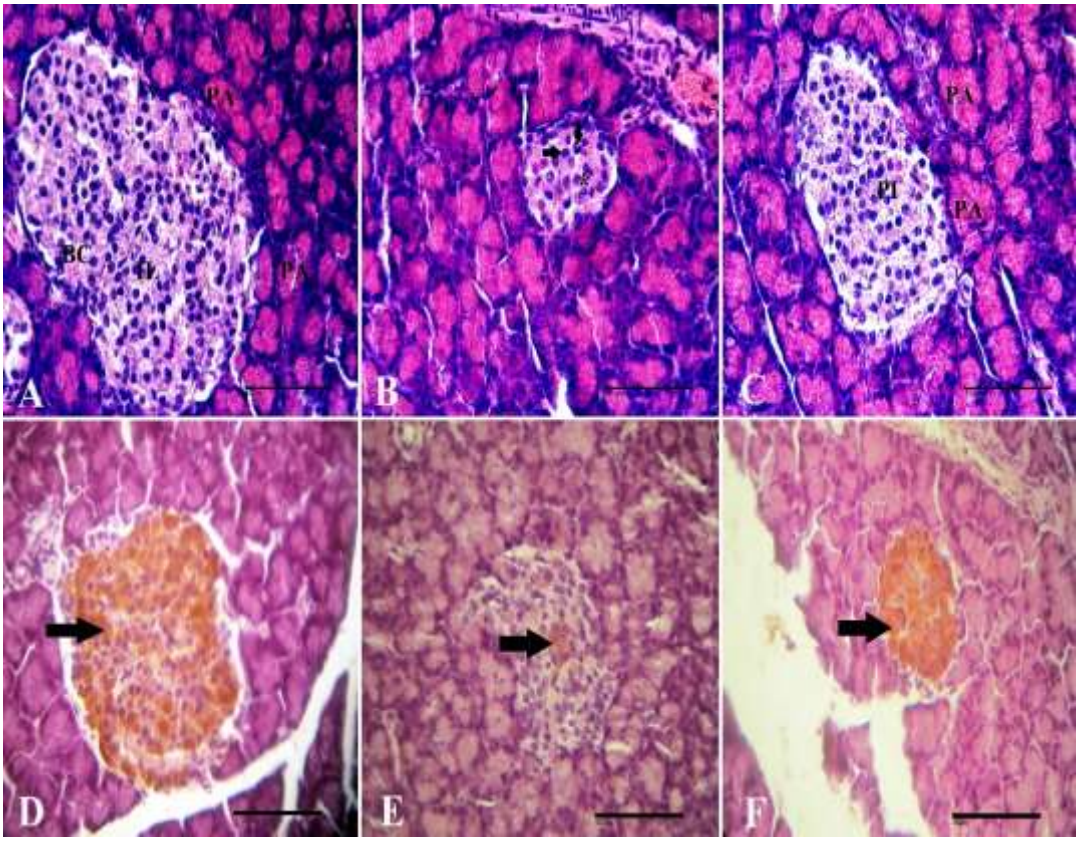

Figure 2: A, photomicrograph of H&E stained rat pancreatic tissue of control group showing normal Islet's of Langerhans (IL) forming cords separated by blood capillaries (BC) together with normal pancreatic acini (PA) with its basal basophilia and apical acidophilia. B, photomicrograph of H&E stained rat pancreatic tissue of streptozotocin treated group showing atrophy of pancreatic Islet's with infiltration of inflammatory cells (arrows) and deposition of amyloid like material (*). C, photomicrograph of H&E stained rat pancreatic tissue of diabetic group treated with stevia showing restoration of normal appearance of pancreatic Islet's (PI) and pancreatic acini (PA). D, photomicrograph of insulin immunostained rat pancreatic tissue of control group showing normal expression of insulin within pancreatic Islet's (arrow). E, photomicrograph of insulin immunostained rat pancreatic tissue of streptozotocin treated group showing mild expression of insulin within pancreatic Islet's (arrow). F, photomicrograph of insulin immunostained rat pancreatic tissue of diabetic group treated with stevioside showing increased expression of insulin within pancreatic Islet's (arrow). Scale bar=50 µm.

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Conclusion

Stevioside showed insulin mimetic activities and regulated diabetes and its associated metabolic alterations at the biochemical, molecular and histochemical levels. Moreover, stevioside restored normal pancreatic cell function as indicated by increase in insulin immune reactivity.

Competing Interest: The authors declared that no conflict of interests.

Authors' Contributions: OMS, NSA, MMS; conceived and designed the experiments. OMS, MMS, AAM; performed experiments. MMS, NSA; analyzed data. MMS; biochemical assays. OMS, MMS, AAM; molecular experiments. MAN; histopathology experiment. OMS, MMS, NSA; interpretations data.OMS, NSA, MMS, AAM, MAN; manuscript revision.

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