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Efficiency of Bioactive Compound Isolated from *Psidium guajava* for Anti-Diabetic Activity on Streptozotocin Induced Diabetic Rats

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ABSTRACT

The present research was designed to investigate the differential expression of specific gene in diabetic rats treated with novel protein isolated from extract of *Psidium guajava* leaves used as trial therapeutic compound for drug development. Streptozotocin- induced diabetic rats used as model to design drug for the treatment of diabetes and its related disease. Administration of a novel protein to diabetic rats significantly decreased the levels of blood glucose, glycosylated hemoglobin and increased body weight gain and biochemical parameters. The diabetic rats were restored to near normal levels by experimental treatment with bioactive compound isolated from *Psidium guajava*. Liver and Kidney tissues from STZ induced diabetic rats were subjected to RNA extraction for gene expression by using real time RT-PCR. Diabetic -specific genes of interest such as IGF-1 was chosen and the expression level of these genes has examined and the abnormal expression of genes in STZ induced diabetic group would be rescued by the bioactive of *Psidium guajava* leaves based therapy. Gene expression of IGF-1 was up regulated after administrated of *Psidium guajava* bioactive evidenced by RT-PCR. Administration of plant based compound to diabetic rats significantly enhances survival, and normal level biochemical process ability to improved function in diabetic animals. The present study reveals the efficacy of *Psidium guajava* extract on streptozotocin- induced diabetic rats in the amelioration of diabetes, which may be attributed to its hypoglycemic property along with its antioxidant potential. The efficacy was compared with a standard hypoglycemic drug glibenclamide.

INTRODUCTION

Diabetes mellitus is classified into two types, insulin dependent diabetes mellitus (IDDM, Type 1) and non-insulin-dependent diabetes mellitus (NIDDM, Type 2). Type II diabetes is characterized by peripheral insulin resistance and impaired insulin secretion. Diabetes causes various cardiovascular complications, which have become the major cause of morbidity and mortality in the diabetic population. Moreover, mortality from cardiac diseases is approximately two- to fourfold higher in patients with diabetes than in those who have the same magnitude of vascular diseases without diabetes, and diabetic cardiomyopathy can occur without any vascular pathogenesis [1]. Several studies have shown that hyperglycemia as an independent risk factor directly causes cardiac damage, leading to diabetic cardiomyopathy [2]. However, mechanisms for the pathogenesis remain unclear [3]. Diabetic hearts, including streptozotocin (STZ)-induced diabetic animal models, displayed a reduction in cardiac mass over time, myocardial hypertrophy and interstitial and perivascular fibrosis at late phase [4]. These late-phase changes are believed to result from early responses of myocardium to suddenly increased glucose levels [5]. Early responses of myocardial cells to hyperglycemia include metabolic abnormalities, subcellular defects, abnormal expression of genes, and, consequently, cardiac cell death [6].

Fasting blood glucose was estimated by the oxidase/peroxidase method [7]. Glycosylated hemoglobin was estimated using the diagnostic kit from Biosystems, Spain. Plasma insulin level was assayed by the Radio Immuno Assay (RIA) kit (Diasorin, Saluggia, Italy), using human insulin as standard. C-peptide level was assayed by the chemiluminescence immunoassay method. Hexokinase and glucose-6-phosphatase were assayed by standard protocols [8]. Serum glucose, cholesterol, triglycerides (TGS), HDL and serum insulin were estimated. Thiobarbituric acid reactive substances (TBARS) (Lipid peroxides) and hydroperoxides were estimated according to method of respectively. Insulin resistance has emerged as an impaired biological response to insulin caused by reduced insulin-stimulated glucose uptake in skeletal muscle and by impaired suppression of endogenous glucose production, which are critical for maintaining normal glucose homeostasis.

Guava (*Psidium guajava* L.), is one of the most common fruit crops grown in most of the agro ecological zone nutrition improving and high yield potential crop. Guava leaves have long been recognized for their antimicrobial activity. *Psidium guajava* L, (Myrtaceae) is one of the parts in folk medicine that has been used for the management of various disease

conditions and is believed to be active. The ethnobotanical studies and folklore claiming reviewed that the leaves of the Guava were used for antioxidant, hepatoprotective, anti-allergy, antimicrobial, antigenotoxic, anti-plasmodia, cytotoxic, antispasmodic, cardioactive, anticough, antidiabetic and anti-inflammatory activities. Guava leaf tea is commonly used as a medicine against gastroenteritis (dysentery) and child diarrhea. WHO (world health organization) says that plants would be the best source for obtaining different types of medicines and drugs. These natural products are widely used by human with its effective results.

The medicinal properties of guava fruit, leaf and other parts of the plant are also well known in traditional system of medicine. Since each part of guava tree possesses economic value; it is grown on commercial scale. With a long course and serious complications often resulting in high death rate, the treatment of diabetes spent vast amount of resources including medicines, diets, physical training and so on in all countries. Thus searching for a new class of compounds is essential to overcome diabetic problems. There is continuous search for alternative drugs[9].

Diabetes can be induced by selective destruction of the insulin-producing β-cells of the pancreas with a single, rapid injection of streptozotocin (STZ), a glucose moiety with a very reactive nitrosourea group from cell, Based on this concept the work present study could designed to analyze and find out a novel drug to treat diabetic condition in the experimental trial of mice model. Streptozotocin (STZ) is an antibiotic that can cause pancreatic *β*-cell destruction, so it is widely used experimentally as an agent capable of inducing insulin-dependent diabetes mellitus (IDDM), also known as type 1 diabetes mellitus (T1DM).

Present research work was performed for screening of bioactive constituents of leaves extract of *Psidium guajava* and the right compound can be identified by using classical biochemical techniques. This study describes protocols for the production of insulin deficiency and hyperglycemia in mice using STZ. These models for diabetes can be employed for assessing the mechanisms of T1DM, screening potential therapies for the treatment of this condition, and evaluation of therapeutic options for plant based reference for anti-diabetic activity.

MATERIALS AND METHODS

Preparation of plant sample:

Psidium guava plant leaves were collected from Sri Adichunchanagiri women's College, Tamil Nadu branch Mutt Cumbum, TheniDt, TN.

Preparation of methanolic extract of *Psidium guava*

Leaves were washed with double distilled water and dried under air and then measured quantity of leaves were cut into pieces, homogenized and extracted with methanol by using soxhlet apparatus extracted cellular contents and the extract was concentrated under vacuum and stored for further pharmacological studies. Freshly prepared crude extracts were qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extracts was performed using the following reagents and chemical: Alkaloids with Dragondroff's reagent flavonoids with the use of Mg and HCI: Tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce stable foam and steroids with Liebermann-Burchard reagent. These were identified by characteristic color changes using standards procedure[12].

Drugs and chemicals*:*

Streptozotocin, Nicotinamide, Glibenclamide were procured from Sigma-Aldrich Labs, GOD-POD Kits were procured from Reckon diagnostics, Total cholesterol and Triglyceride kits were procured from Excel diagnostics Pvt Ltd, Hyderabad

Experimental design

Experimental Animals: Wistar rats of both sexes weighing 150-200 gm were used for study (Mahaveer Enterprises, Hyderabad). All animals were maintained under standard laboratory conditions (temperature 22±2^{0}C and humidity 50± 15%) with 12 hours day: 12 hours night cycle. The animals were fed with normal laboratory diet and allowed to drink water ad libitum. All protocols were performed in accordance with the Institutional Animal Ethical Committee (IAEC) as per the directions of the CPCSEA (Committee for the purpose of Control and Supervision of Experiments on Animals). Experiments were performed on male Wistar rats from the Animal House of the Madurai Kamaraj university, Madurai, Tamilnadu.

Induction of Diabetes Mellitus:

Streptozotocin (STZ) was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in normal saline. Diabetes was induced in overnight fasted Wistar strain albino rats by a single intraperitoneal injection of 60 mg/kg streptozotocin, 15 min after the i.p. administration of 120 mg/kg of nicotinamide. Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined at 72 h after administration. Rats with fasting blood sugar levels around 160 to 300 mg/dl were selected for the study. The blood glucose concentration was measured every week from the day of STZ injection. The blood samples were collected from the tail vein once a week and the blood was deproteinized. The obtained supernatant was used immediately for the determination of blood glucose by glucose Oxidase/peroxidase method spectrophotometrically[10]. Streptozotocin was obtained from Sigma Chemicals Co., St. Louis, MO, USA.

Experimental Design for Hypoglycemic activity and Oral glucose Tolerance Test:

The animals were divided into four groups (n=6) Group-1: Rats served as normal-control-received 0.9% Saline. Group-II: Rats served as Standard-received Glibenclamide (10 mg/kg b.wt). Group-III: Rats were administered methanolic extract of *Psidium guava* plant leaves (100mg/kg b.wt) as a fine suspension orally. All animals were fasted for 18 hr, before experimentation, but allowed free access to water. Blood samples were collected for the measurement of blood glucose level by puncture of retro-orbital plexus at 0hr, 2hr, 4hr and 6hr from control and test group animals after feeding the plant extract.

Biochemical analysis

The body weight of all rats was measured at days 0 and 20. Blood samples were collected from the tail vein of the experimental animals at 0, 5, 10,15 and 20 days. In all animals, blood samples were collected from retro-orbital plexus under light ether anesthesia and centrifuged at 2500 rpm for 20 minutes to separate serum. Blood glucose was estimated by O-toluidine method [11]. The levels of glycosylated hemoglobin were estimated using the diagnostic kit from Biosystems, Spain [2].

The liver and Kidney tissues were excised, rinsed in ice-cold saline and then homogenized in Tris–HCl buffer (pH 7.4). The tissue homogenates were used for the following experiments Protein was estimated by the method [13] and total RNA content was prepared.

Extraction of total RNA:

The kidney and liver tissue were homogenized with RNA ZOL (2ml per 100mg tissue) with few strokes in a glass Teflon homogenizer. The use of guanidium to lyse cells was originally developed to allow purification of RNA from cells rich in endogenous ribonuclease. Guanidium denatures protein and thus inactivates any ribonucleases were present. RNA Extraction was carried out 0.2ml Chloroform was added to 2ml of tissue homogenate and the samples were tightly closed and shaken vigorously for 15 seconds and let them stay on ice for 15 minutes. The suspension was centrifuged at 12,000 rpm (-4ºC) for 15 minutes. RNA precipitation step was continued transfer of the aqueous phase to a fresh tube, added an equal volume of isopropanol and stored the samples for 45 minutes at -20ºC. Samples were centrifuged for 15 minutes at 12,000rpm (-4ºC). RNA precipitate (often invisible before centrifugation) formed a white pellet at the bottom of the tube. The supernatant was removed and washed the RNA pellets twice with ice cold 75% ethanol by vortexing and subsequent centrifugation for 8 minutes at 12,000 rpm (-4ºC) and the pellets were dried under vacuum for 10-15 minutes. The RNA pellet was dissolved in 1mM EDTA, pH 7.0. Diethyl pyrocarbonate (DEPC) treated and RNase free solutions should be used for RNA solubilization. The final preparation was free from DNA and protein and pure RNA samples checked by measuring its OD at 260 nm. The preparation was ready for dot blot hybridization gel electrophoresis to detect specific mRNA by Northern blotting. RNA blot RNA was determined by hybridizing the membrane to a specifically labeled DNA probe[14]. The RNA ZOL method can be completed within 3 hours providing both high yield and purity of RNA preparation. RNA isolated with the use of RNA ZOL is non-degraded, free of DNA and protein and contains the whole spectrum of RNA, molecules, including small RNAs.

Real Time Reverse Transcriptase-PCR (RT-PCR)

The assay performs using a one-step RT-PCR kit (Qiagen, Germany). A set of primer were designed for real-time RT-PCR assay. The sequences (5′ to 3′) for the primer pairs and their product lengths (bp) are mentioned below:

IGF-1 (f) AGGCTATGGCTCCAGCATTC

IGF-1 (r) AGTCTTGGGCATGTCAGTGTC

ANG1 (f) GACACCTTGAAGGAGGAGAAAG

ANG1 (r) GTGTCCATGAGCTCCAGTTGT

VEGF (f) ACCCCGACGAGATAGAGTACAT

VEGF (r) CTTC TAATGCCCCTCCTTGT

The reaction contained 5 µl of RNA, 0.6 µM of each primer, 0.2 µM of the Taq Man probe. Real time-PCR machine was used with the following thermal steps: reverse transcription 30 min, initial denaturation, followed by 40 cycles of denaturation, annealing, and extension (RT-PCR kit Qiagen, Germany). Gel Electrophoresis was carried out for the assessment of PCR products using Agilent Bioanalyzer system. The PCR product was visualized as a single compact band with expected size (IGF-1:166bp; ANG1:143bp; VEGF: 200bp).

RESULTS AND DISCUSSION

Methanolic extract of *Psidium guajava* was screened to identify compound of therapeutic potential source for the drugs against human illnesses. Extract of *Psidium guajava* was contributed different compounds. Methanolic extracts showed the presence of maximum Secondary metabolites such as Alkaloids, Flavonoids, Glycosides, Saponins, Phenols, Tannins, Steroids, and Aminoacid. The detailed result was shown in table 1.

Table 1: Phyto-Chemical analysis

Sr. No.	Secondary metabolites	Extract
1	Alkaloids	+
2	Flavonoids	+
3	Glycosides	+
4	Saponins	+
5	Phenols	+
6	Tannins	+
7	Steroids	+
8	Amino acid	+
9	Diterpenes	-
10	Anthraquinones	-

(+ indicates presences,- indicates absences)

In diabetic condition, the excess of glucose present in the blood reacts with hemoglobin to form glycated hemoglobin, which has altered affinity for oxygen and this may be a factor in tissue anoxia. Glycated hemoglobin was significantly increased in diabetic rats, and this increase was directly proportional to fasting blood glucose **[Figure1].**

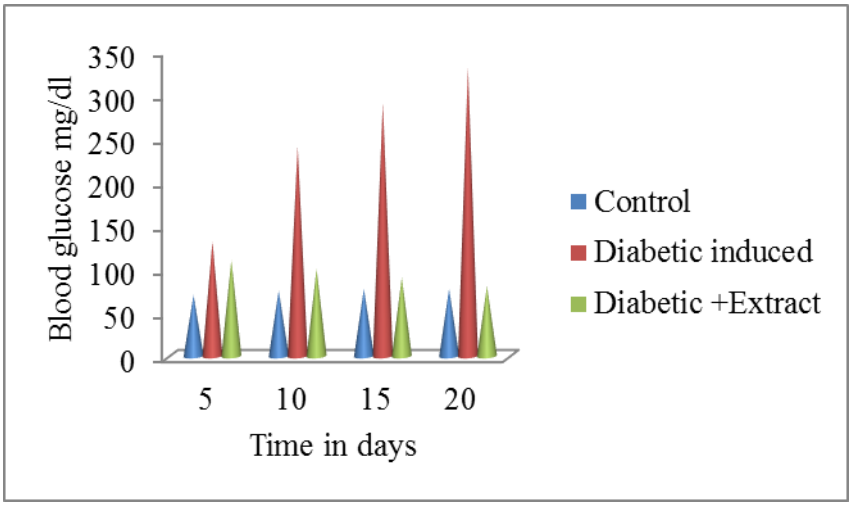

Figure 1: Analysis of blood glucose in STZ induced diabetes

Legend: Figure shows the level of blood glucose at different intervals after diabetic induction at *Psidium guajava* extracts experimental animals. X- Indicates experimental period in days. Y- Indicates blood glucose level.

There was increasing evidence that advanced glycation end products (AGEs) play a pivotal role in atherosclerosis, in particular in diabetes. Figure 2 revealed AGE accumulation is a measure of cumulative metabolic and oxidative stress and also increased AGE accumulation was closely related to the development of cardiovascular complications in diabetes [15]. The receptor for advanced glycation end products (RAGE) was a cell surface receptor whose signaling pathway has been implicated in atherogenesis. Hemoglobin was highly susceptible to non-enzymatic glycation, Glycated hemoglobin was an effective means to screen for diabetes. The decreased level of total hemoglobin in diabetic rats is mainly due to the increased formation of glycosylated hemoglobin (HbA1c). HbA1c was found to increase in patients with diabetes mellitus and the amount of increase is directly proportional to the fasting blood glucose level. During diabetes, the excess glucose present in the blood reacts with hemoglobin to form HbA1c) HbA1c was used as a marker for estimating the degree of protein glycation in diabetes. Data shows **[Figure 2]** that glucose is not only the main energy source for short periods but also the major sources of diabetes mellitus complications, mainly

by forming oxidative and proinflammatory advanced glycation end products (AGE). Further studies were needed to identify the gene expression behind the injection of active principles of the plant extract.

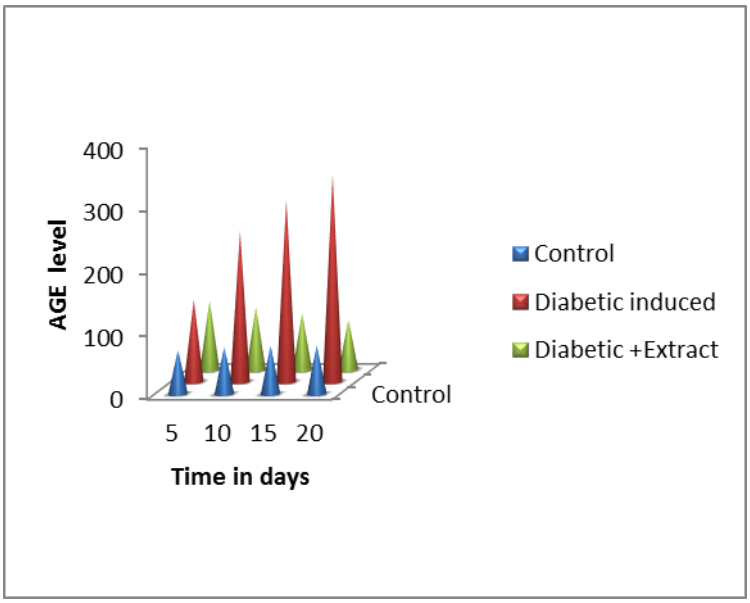

Figure 2: Level of AGE experimental animal

Legend: figure 2 showed the level of AGE products at different time interval after diabetic induced *Psidium guajava* leaves extract treated animals. X- Indicates experimental period in days. Y -Indicates AGE level.

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 to 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 2:1 ratio (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.

A single injection of STZ is widely used to generate a rat model of type I diabetes, which results from the selective toxicity of STZ towards the insulin-producing β-cells in pancreatic islets [16]. A number of factors influence the vascular dysfunction that develops in this model, such as the age of the rats, the dose of STZ administered and the duration and severity of hyperglycemia [17].

STZ-induced diabetes may serve as a model of the type 1 diabetes of human and is associated with severe hyperglycemia in combination with hypoinsulinemia and ketoacidosis. In experimental animal models of STZ-induced diabetic cardiomyopathy, multiple studies demonstrated myocardial atrophy as opposed to hypertrophy with loss of heart weight, reduced cardiomyocyte transverse diameter, loss of contractile proteins and cardiomyocyte dropout. Moreover, in recent studies using myocardial biopsy materials from patients with diabetes without hypertension, smaller diameter of cardiomyocytes was observed than in controls without diabetes and hypertension. Cellular mechanisms of diabetes-triggered cardiac atrophy are not clearly understood. Calorie deprivation associated with metabolic disturbance in diabetes and energy production shifted from glucose utilization towards β-oxidation of free fatty acids may cause atrophic alterations in the myocardium.

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 were 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[18], demonstrated many changes occurred in their gene expression. More than a half of gene analyzed (565 from total 835 genes) showing twofold 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. In this study, four specific diabetes genes were chosen to present the hypoglycemic effect of the

extract. Treatment with Protein extracted from *Psidium guava* plant leaves may show significant changes in rescue the abnormal expression of genes in STZ induced diabetic rat.

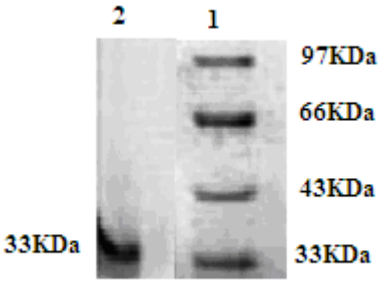

Figure 3: Showed the separated active compound on 10% SDS Gel

Figure 3 showed the various fractions collected from chromatography separations of *Psidium guajava* extract and samples were pooled then purified protein compounds analyzed by HPLC. The data confirmed the presence of novel molecules which has size as33KDa protein and its potential properties exhibited when administrated into experimental animals.

Gene-expression analysis of angiogenic and cardiac markers was done for all groups of protein from *Psidium guava* leaves extract treated, whereas nontreated groups were used as controls. Elevated levels of VEGF, ANG-1 (angiogenic markers) were observed in protein injected groups compared with all another non treatment group. Molecular studies can be assessed by measuring the quantity gene expression of the final product of protein. However, measuring RNA level was critically important to have a good quality of RNA in **Figure 4**. RNA assessment was carried out to ensure the reliability of the RNA samples isolation technique yields good quality of RNA. The intact total RNA sample has shown distinct 18S and 28S subunit spikes, with 2:1 ratio (28:18S).

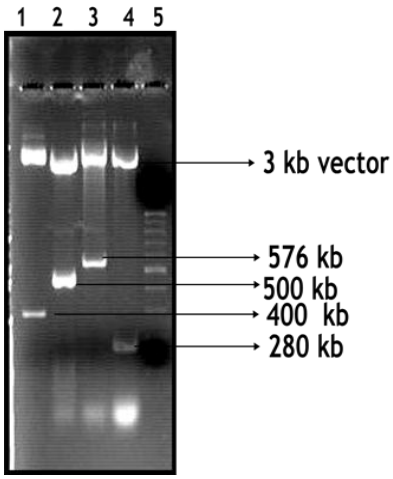

Figure 4: Probes used for Northern Blot Analysis (2% Agarose gel)

Lane 1: ANG

Lane 2: MLC2

Lane 3: ANF

Lane 4: β-MHC

Lane 5: 100 bp Ladder

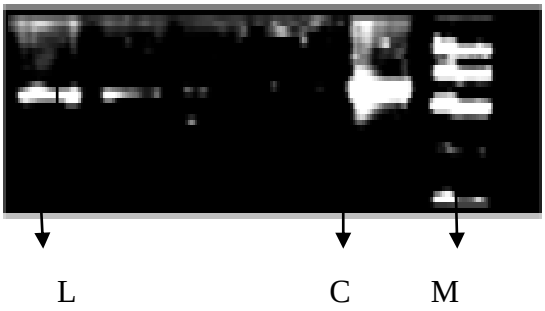

Figure 5: RT- PCR to identify the reporter gene expression in Extracted protein injected rats

Legend: Gene-expression analysis ofdiabetic ratspretreated withprotein from *Psidium guava*extract and Kidney tissues subjected to treatment in the presence of different concentrations of IGF-1:166bp. Lane 1 -5 indicates various concentrations of 0.06ng/ml, 0.07ng/ml, 0.08ng/ml, 0.09ng/ml and 0.1ng/ml IGF-1.

In studies of target gene expression by RT-PCR, the use of internal reference genes were 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 and to make sure the amplified product within the expected size of gene profiling analysis on STZ induce diabetic rat similar observation by [19] demonstrated many changes occurred in their gene expression of specific genes were chosen to analysis the hypoglycemic effect of the extract treatment with protein from *Psidium guava* leaves extract might show significant changes in rescue the abnormal expression of genes in STZ induced diabetic rats **Figure 5**. Etiologically, the main cause responsible for the development of heart dysfunction was sustained hyperglycemia which promotes the formation of advanced glycation end products [20]. Protein purified from *Psidium guava* leaves extract could be used as a formulating drug in discriminating diabetic animals.

CONCLUSION

Psidium guajava. extract have potent antidiabetic effects in the treatment of STZ induced diabetic in rat and compared with standard glibenclamide was effective in moderate diabetic rats not in severe diabetic animals. However, the purified protein effect of the extracts was more potential than glibenclamide. Moreover, elucidation of the mechanisms concerning diabetes mellitus contributes to the prevention of metabolic syndrome and the appearance of effective new medications available to treat diabetic cardiomyopathy formulated as drugs that could be used by clinicians in the treatment of their diabetic patients.The diabetic rats were restored to near normal levels by experimental treatment with bioactive compound isolated from *Psidium guajava*. The bioactive compound present in the extract will be characterized the right principle compound formulated as drug for diabetic condition after clinical trial with animal model reference to plant based cheap pharmaceutical product in our research work in future.

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