Metabolic Effects of the Consumption of Aqueous *Kalanchoe pinnata* Preparation in Streptozotocin-Induced Diabetic Rats

By

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ABSTRACT

Background: *Kalanchoe pinnata* is a medicinal plant found in South America, India, and the Caribbean. Aqueous preparations of the leaves are traditionally used for the treatment of a wide range of diseases in many parts of the world, including diabetes. While previous studies have confirmed the acclaimed hypoglycemic properties, its metabolic effects in diabetes remains unknown. In this study, we evaluated the metabolic effects of *K. pinnata* preparation in the management of streptozotocin-induced diabetic rats.

Methods: Eighteen (18) adult Sprague rats were assigned by weight into three groups for a 30 day study [six rats per group, average body weight (297.28 ± 15.17 g)]. The groups were composed as follows: Healthy rats receiving de-ionized water (Normal Control); diabetic rats administered de-ionized water (Diabetic Control); diabetic rats administered aqueous preparation of *Kalanchoe pinnata* leaves (~ 0.14 grams / kg body weight; Diabetic Treated). Animals were euthanized by decapitation on day 30 after the commencement of the feeding trial. Blood and organ samples were collected for the biochemical and hematological assays.

Results: The diabetic groups lost weight and consumed less food compared to the normal control group. However, the diabetic treated groups' loss in weight was more evident than the diabetic control groups' loss in weight. We noted a significant ($P < 0.05$) decrease in blood glucose level in the diabetic group treated with *K. pinnata* preparation compared to the other groups. Serum albumin was significantly ($P < 0.05$) elevated in the diabetic treated group compared to the diabetic control. Serum cholesterol and triglyceride levels were significantly ($P < 0.05$) reduced in the diabetic treated group

compared to the diabetic control. There was a significant ($P < 0.05$) increase in serum alkaline phosphatase (ALP) activity in the diabetic control and diabetic treated groups compared to the normal control group. Serum blood urea nitrogen (BUN) level was significantly ($P < 0.05$) elevated in the diabetic treated group compared to the other groups. Liver lipid profile was not significantly altered among the groups. However, *K. pinnata* administration significantly ($P < 0.05$) reduced renal total cholesterol level compared to the diabetic control. Serum antioxidant enzyme activities were increased and lipid peroxidation was reduced in the diabetic treated group compared to the diabetic control group. There was a significant ($P < 0.05$) decrease in serum amylase activity in the diabetic control and improved amylase activity in the diabetic treated group. We observed a significant ($P < 0.05$) increase in magnesium ATPase activity in the intestinal mucosa and erythrocyte membrane of the diabetic treated group compared to the diabetic control. We also noted a significant ($P < 0.05$) increase in the hepatic pyruvate kinase activity in the diabetic treated group compared to the diabetic control group. Renal $NADP^+$-isocitrate dehydrogenase activity was significantly ($P < 0.05$) increased in the diabetic treated group compared to the diabetic control group. Serum IL-6 level was significantly ($P < 0.05$) reduced in the diabetic treated group compared to the diabetic control group.

Conclusion: Overall, the consumption of aqueous *K. pinnata* preparation may increase pyruvate kinase activity with subsequent increase in hepatic glucose utilization for energy generation and decrease in blood glucose level. Additionally, we also noted that interleukin-6, which was significantly elevated in the diabetic control group, was reduced to normal control level due to the treatment. This may be indicative of reduced

inflammation and reduced chances of vascular disease. However, the observed increase in serum ALP activity and BUN level may be indicative of hepatic or renal damage. Further studies are needed to evaluate the long-term effect of *K. pinnata* preparation consumption on serum ALP activity and BUN level.

Keywords: diabetes, herbal remedies, *Kalanchoe pinnata*, cardiovascular disease, oxidative stress

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1. INTRODUCTION

1.1. Diabetes Mellitus

Diabetes is a metabolic syndrome characterized by hyperglycemia resulting from alterations of insulin secretion or action. It can occur when the body produces little insulin, ceases to produce insulin, or becomes progressively resistant to the action of insulin [1]. In 2003, the Expert Committee on Diabetes reformed the World Health Organization classification scheme, and defined five types: Type 1, a T-cell mediated autoimmune disorder that results in the destruction of β-cells of the islets of Langerhans in the pancreas [1]; Type 2 diabetes, the most prevalent form of diabetes, results from the development of insulin resistance by the cells due to age, obesity and lack of physical activity. The third type, gestational diabetes, occurs when hormonal changes during pregnancy result in the development of insulin resistance. Other types of diabetes result from genetic defects of β-cells, or defects of insulin action [1]. Figure 1 details the common differences between Type 1 and Type 2 diabetes.

Table 1
Distinguishing Characteristics for Type 1 and Type 2 diabetes

Characteristics	**Type I**	**Type II**
Usual age of onset	<30	> 40
Nature of onset	Rapid	Slow
Symptoms	Polyuria, Polydipsia, polyphagia	May be asymptomatic
Signs	Wasting, dehydration, loss of consciousness	Commonly obese
Tendency to ketosis	Very Prone	Little
Response to insulin	Sensitive	Commonly Resistant
Treatment: 1. Diet 2. Insulin 3. Sulfonylureas	 Insufficient Essential Ineffective	 May be sufficient Needed in some cases May be effective

Adapted from Newsholme and Leach [2] .

Type 1 diabetes (formerly known as insulin-dependent diabetes or juvenile onset diabetes) is a life-threatening, multisystem, metabolic disease of abrupt onset characterized by severe insulin deficiency as a result of autoimmune destruction of insulin producing pancreatic beta cells [3]. In humans, the beta-cells can be damaged by a combination of genetic, environmental and nutritional (ingestion of cow's milk in early childhood) factors, viral infections (e.g., Mengo virus 2T), or autoimmunity. Therefore, individuals with Type 1 diabetes are dependent on exogenous insulin for survival [4]. Without proper treatment, lack of cellular glucose will stimulate lipolytic and even proteolytic activities in order to provide cellular energy. This shift can result in chronic hyperglycemia (i.e., increased blood sugar), systemic acid-base imbalance, insufficient

glucose delivery to the brain and retina, inadequate blood supply to the tissues, widespread vascular degeneration, and neuropathy [4].

Type 2 diabetes mellitus (formerly known as non-insulin dependent diabetes or adult-onset diabetes) is more common (about 90% of diabetic patients) than Type 1 diabetes mellitus. It results from a combination of insulin resistance and relative insulinopenia. While the study uses a Type 1 model for analysis, the results of the study can be extended to type 2 patients since both occur due to a lack of insulin. Until recently, Type 2 diabetes mellitus was observed almost exclusively in people over age 40. However, with the growing epidemic of obesity in children and young adults, Type 2 diabetes mellitus can now be observed in young children [5]. The symptoms of Type 2 diabetes mellitus are usually mild and take several years to develop. In most cases diabetic complications (e.g. diabetic retinopathy, peripheral neuropathy, diabetic nephropathy and cardiovascular disease) are present during diagnosis. The development of Type 2 diabetes mellitus has been linked to the synergistic effects of genetic susceptibility and obesity. Women are affected more often than men predominantly because of higher rates of obesity and lower lean body mass. Lack of treatment could result in the secondary complications found in Type 1 diabetes mellitus.

Gestational diabetes is a state of glucose intolerance that develops in response to the rapid influx of blood glucose in women during pregnancy. It occurs in 2-9% of pregnancies and usually presents after the 28^{th} week [6]. The risk of developing gestational diabetes is increased in (a) women over the age of 25, (b) women under the age of 25 and obese, (c) women with a family history of diabetes in first-degree relatives, and (d) women belonging to a high risk ethnic or racial group [4]. The causes of gestational diabetes are

multifactorial and complex. The Pedersen hypothesis that 'maternal hyperglycemia results in excess transfer of glucose to the fetus resulting in fetal hyperinsulinemia' has formed the basis of understanding the development of gestational diabetes [7]. Obesity and gestational diabetes are known to produce insulin resistance, diverse abnormalities in oxidative stress, protein glycation, and decreased cellular processes, which results in impaired endothelial and vascular function that can cause abnormalities in placental function leading to increased fetal morbidity and still birth [6]. Glucose regulation often returns to normal levels after birth; however, up to 40% of mothers develop Type 2 diabetes mellitus later in life [4].

For this study, Type 1 diabetes was induced by injecting streptozotocin (STZ) intraperitoneally. STZ is a glucosamine–nitrosourea compound derived from *Streptomyces achromogenes* that specifically targets pancreatic beta cells [8]. STZ is able to enter and accumulate in the beta cells using the same GLUT2 cell entry pathway that is utilized by glucose [9]. Once inside the cells, it destroys the beta cells and results in the development of diabetes. GLUT2 receptors are expressed specifically in pancreatic β-cells but not in pancreatic acinar cells [10]. Tissues like cardiac and skeletal muscle express GLUT1 and GLUT4 receptors but do not accumulate STZ [10]. However, STZ may also be toxic to the liver because GLUT2 receptors are also expressed in this organ [11].

1.2. Epidemiology and Associated Complications of Diabetes Mellitus

Type 1 and 2 diabetes mellitus are by far the most common endocrine disorders with a worldwide prevalence rate of about 5% [12]. In the US, diabetes is the seventh primary

cause of death. According to new estimates from the Centers for Disease Control and Prevention (CDC), about 26 million Americans are currently diabetic [3]. Additionally, an estimated 79 million US adults are pre-diabetic, a condition in which blood sugar levels are higher than normal, but not high enough to be diagnosed as frank diabetes. The current estimate from CDC shows that 1 in 10 Americans have diabetes, but the number could grow to 1 in 5 or even 1 in 3 by mid-century if current trends continue [13]. Almost half of diabetes-related deaths occur in people under the age of 70 years; with 55% of these deaths occurring in women. The World Health Organization (WHO) projects that deaths caused by diabetes will increase by more than 50% in the next 10 years if there is no urgent action [14]. Microvascular and macrovascular complications are the major cause of morbidity and mortality in patients with the disease [15]. Especially in diabetic patients older than 65, heart disease is the major cause of death in 68% of the individuals [16]. Retinopathy and nephropathy are the most common complications of Type 1 diabetes mellitus. In diabetic retinopathy, excess glucose blocks capillary blood flow behind the retina and causes the capillaries to bulge and form pouches [17]. Excessive pressure causes the capillaries to fail, allowing fluid to leak out into the macula, where focusing occurs in the eye, and results in macular edema [17]. After several years, these blood vessels can become so damaged that they close off, prompting new blood vessels to be produced [17]. However, the new vessels are weak, leaking blood into the vitreous humor, resulting in vitreous hemorrhages, and blocking vision [17]. Diabetic retinopathy is the leading cause of acquired blindness, and has become one of the most feared complications among young adults [3]. Renal disease is a major complication of Type 1

diabetes mellitus, resulting in a high percentage of morbidity and mortality and producing significant costs for the healthcare system [3].

1.3. The Effect of Diabetes on Cellular Metabolism

The normal human pancreas contains specific regions called islets of Langerhans that contain insulin expressing beta-cells, glucagon expressing alpha-cells, and somatostatin expressing gamma-cells. These pancreatic beta cells respond to small changes in extracellular glucose concentrations. Glucose is transported into beta-cells via high capacity glucose transporters (GLUT1, 2, and 3 in humans), enabling rapid detection of extracellular and intracellular glucose concentrations. Once inside the beta-cell glucose is phosphorylated by glucokinase which acts as a 'glucose sensor' and couples insulin secretion to the level of glucose detected. The regulation of whole body fuel homeostasis primarily involves insulin action in skeletal muscle, adipose tissue, and liver where insulin promotes uptake and storage of carbohydrate, fat, and amino acids, while at the same time antagonizing the catabolism of these fuel reserves.

In normal conditions, plasma glucose stimulates glucose uptake by the liver, kidney, and skeletal muscle to produce cellular energy and suppresses glucose production. Figure 1 diagrams the major sources of blood glucose as well as the common metabolic pathways. Extracellular glucose that is not absorbed will be transported in the blood and secreted as urinary glucose. However, when glucose enters hepatic or non-hepatic tissue, it can be converted to glycogen (in hyperglycemic conditions) for long term storage or it undergoes glycolysis (in hypoglycemic conditions) and is converted into pyruvate and energy. In extreme hypoglycemic conditions, hepatic enzymes resort to breaking down amino acids into glucose for cellular energy.

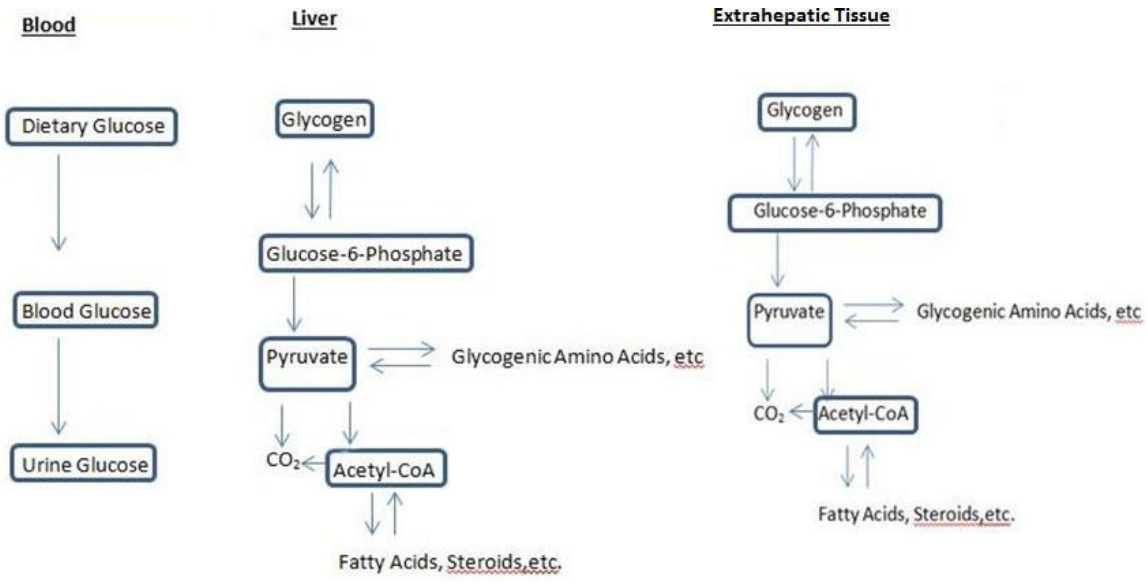

Fig. 1 Major Sources and Fates of Blood Glucose in the Body. Adapted from Wood [18].

In severe diabetes, patients are unable to metabolize glucose efficiently and cannot synthesize fatty acids and triglycerides from carbohydrates or amino acids due to the failure of insulin secretion or action. Since the cells are unable to detect and absorb the plasma glucose, the activities of glycolytic, lipogenic and pentose phosphate pathway enzymes are suppressed while gluconeogenic, glycogenolytic and lipolytic activities are elevated and thus reversing the normal metabolic pathway illustrated in Figure 2. Instead of glucose being broken down using oxidative decarboxylation and the citric acid cycle, fats are converted into acetyl-CoA, which is then turned into citrate, which enters the citric acid cycle and produces energy.

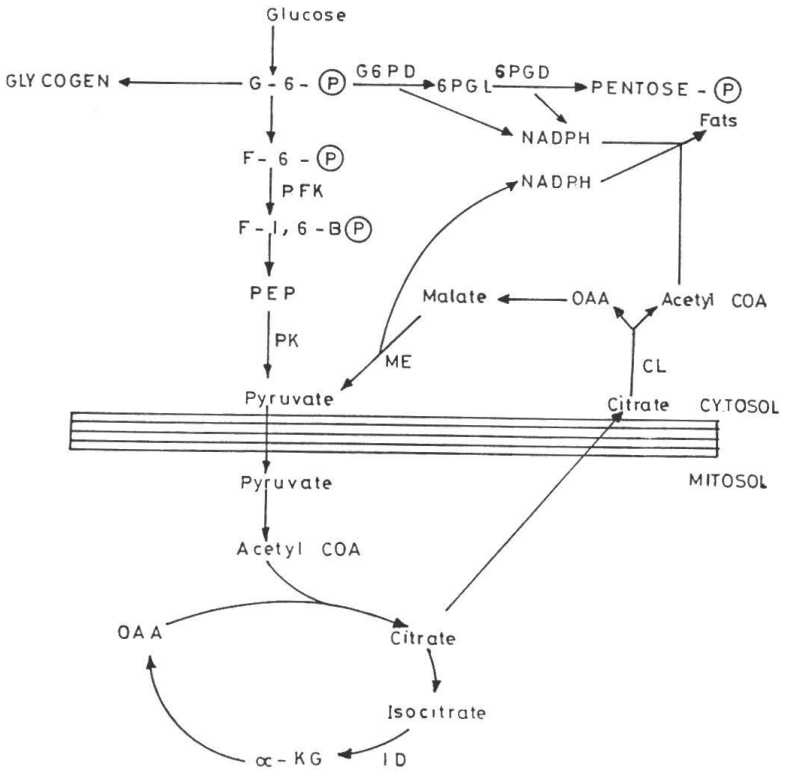

Fig. 2 Summary of the Steps Involved in Carbohydrate and Lipid Metabolism in Non-Diabetic Individuals. Adapted from Omoruyi [19].

The aforementioned disruption of normal carbohydrate and lipid metabolism are not the only side effects of diabetes. Current research indicates that there is a close relationship between obesity and the risk of diabetes. The hormone lipase, which is a principle regulator of free fatty acid release from adipose tissue, is exquisitely sensitive to insulin [20]. In normal individuals, glucose secretion after a meal stimulates insulin secretion which acts to inhibit lipolysis. However in the diabetic state, free fatty acid production increases and lipolysis elevates the fatty acids in the blood system. Elevated fatty acids

levels are also known to further impair peripheral glucose uptake and increase production of ketones which leads to ketoacidosis.

Additionally type 2 diabetes is associated with a high risk of cardiovascular disease (CVD). Since cholesterol is insoluble, it is transported in the circulatory system as a HDL or LDL. LDL cholesterol is highly atherogenic and contributes to the hardening of arteries by aiding the formation of atheromatous plaques [21].Diabetic dyslipidemia consists of moderate elevation of triglycerides, low HDL cholesterol values and small dense LDL particles [22]. This pattern is associated with insulin resistance and is present even before the onset of diabetes. Development of this condition in diabetic patients leads to atherosclerosis and a higher risk for cardiovascular disease.

Finally the alteration of the membrane lipid composition is another consequence of diabetes. Flexibility of the cell membrane is determined by the ratio of (poly) unsaturated to saturated fatty acyl-chains of its phospholipids, which influences the effectiveness of glucose transfer by the GLUT receptors [23]. During the pre-diabetic stage there is a shift from unsaturated to saturated fatty acyl chains of membrane phospholipids which directly causes a decrease in glucose effectiveness and insulin sensitivity [23]. Additionally, a concomitant increase in stiffness of both plasma and erythrocyte membranes may decrease microcirculatory flow, leading ultimately to tissue hypoxia, insufficient tissue nutrition, and diabetes-specific microvascular pathology [23].

Treatment for diabetes mellitus acts to restore the normal metabolic pathways and reverse the dyslipidemia caused by the disease. Traditional medication involves a combination of oral glycemic agents, daily insulin injections, regulation of diet, or physical activity.

Exercise, in particular, has been shown to improve insulin sensitivity and can acutely lower blood glucose in diabetic patients, as well as improve cardiovascular status.

1.4. The Shift from Modern Medicine to Herbal Remedies

The cost associated with treatment of diabetes is expensive and increases exponentially if there are complications. According to Consumer Reports Health, the average diabetic patient spends about $6,000 annually for the management of diabetes [16]. The above figure includes monitoring supplies, medicines, doctor visits, annual eye exams and other routine costs but excludes costs of treatment for associated diabetic complications. Diabetic patients on average pay twice as much as those without the illness for health care. It is projected that deaths caused by diabetes mellitus will increase by more than 50% in the next 10 years if there is no urgent action [13]. About 50% of all patients with Type 2 diabetes mellitus already have signs of at least one diabetic complication at the time of diagnosis [24]. Diabetes care will cost patients up to $3.4 trillion in the next 10 years, and almost 60% of that cost will be paid by the US government [25]. In South Texas, the proportion of uninsured diabetic patients is believed to be a huge burden on the regional economy; media reports state that 17% of the population of Nueces and San Patricio counties suffer from diabetes [26,27].

Clearly, there is an urgent need to find a potent and cost effective option to the current treatment. Millions of people all over the world have resorted to the use of medicinal plants for the management of the disease due to the rising cost of orthodox treatment and associated side effects. Most of these medicinal plants are used in developing countries for the treatment of diabetes, especially in the underprivileged populations. Since

diabetes is a global disease, more than 800 medicinal plants around the world have been identified as possible treatment options. Unfortunately, the use of most medicinal plant supplements in the management of diabetes and other diseases lack scientific backing. For example, doses and side effects of many medicinal plant preparations are not known. This makes herbal medicine a much riskier alternative to modern medicine.

1.5. Kalanchoe pinnata Preparation as a Treatment Option

Since diabetes is a global disease, each country has developed its own traditional remedies in the treatment of diabetes. While some treatments have yielded beneficial effects, there is still a great need to find a comprehensive herbal solution for the treatment of diabetes. In order to identify such treatment options, the resolution of the 31st WHO Assembly requested a complete inventory, evaluation of the efficacy, safety and standardization of medicinal plants. *K. pinnata* is one such medicinal plant found in South America, India, and the Caribbean. It has tall hollow stems, dark green scalloped leaves with red edges and produces dark bell-like pendulous flowers (Figure 3). The smaller reddish leaflets on the edges of the leaves are rooting vegetative buds that are capable of producing individual plants on their own [28]. Aqueous preparations of the plant leaves and roots are used to treat a variety of ailments ranging from diabetes, hypertension, kidney dysfunction, urinary problems and Leishmaniasis [29]. Most of these effects, including the hypoglycemic activity have been validated by research. However, the specific molecular mechanism by which these preparations induce the hypoglycemic response has not been fully elucidated.

Fig. 3 Typical *Kalanchoe pinnata* Plant with Flowers. Adapted from Raintree Tropical Plant Database [28]

Previous studies have suggested several active components of *K. pinnata* that may be responsible for its medicinal activity. For example, flavonoids are compounds that are commonly found in plants and contribute to the antioxidant activities of many plants. The two major flavonoids found in *K. pinnata* leaves are quercetin (3-O-α-L-arabinose (1→2) α-L-rhamnopyranoside), a phenolic acid and quercitrin (quercetin 3-O-α-L-rhamnopyranoside), a well-known dietary phenol [30]. Quercetin has been specifically implicated in the reduction of reactive oxygen species and free radicals [30]. Another flavonoid isolated from the plant, quercitrin works with quercetin in order to inhibit mast cell activation and the development of allergic inflammation [31]. Studies have also identified quercitrin to be a potent anti-diabetic therapeutic agent [32]. It is believed that

quercitrin works in conjunction with quercetin to stimulate beta cell activity and induce insulin release.

In this study, the effect of the preparation on diabetic indices, the integrity of organs, and various metabolic processes were investigated. The overall goal was to establish the gross implication of consumption of *K. pinnata* aqueous preparation in the management of diabetes. To achieve this objective, intestinal and inflammatory responses, blood glucose and lipid profile, oxidative stress, and renal and hepatic glucose metabolism in STZ-induced diabetic rats administered *K. pinnata* preparation was determined with a view to establishing the potential metabolic effects of the preparation.

1.6. Objectives

Objective 1: To investigate the effects of *K. pinnata* aqueous preparation on glucose utilization, digestive enzymes and intestinal ATPase activities in STZ-induced diabetic rats. The goal of this objective was to establish the extent to which food substances are digested to absorbable products in the lumen and the potential effect of *K. pinnata* preparation on intestinal glucose absorption.

Objective 2: To determine the effects of the *K. pinnata* preparation on blood lipid profile, lipid peroxidation and antioxidant enzyme activities in STZ-induced diabetic rats. This information would provide knowledge on the effect of the aqueous preparation on blood lipid distribution and probable role of the plant preparation in the prevention of oxidative damage.

Objective 3: To evaluate carbohydrate and lipid metabolic enzyme activities in the liver and kidney of STZ-induced diabetic rats administered the aqueous preparation of *K. pinnata* and assess organ function. This aim would help to elucidate the role of *K. pinnata* preparation in various metabolic pathways in the liver and kidney.

Objective 4: To determine the complete blood count and inflammatory responses in STZ-induced diabetic rats administered the aqueous *K. pinnata* preparation. This objective would aid in assessing the effect of *K. pinnata* preparation on endothelial function and interleukin levels.

2. MATERIALS & METHODS

2.1. Locations and Animal Observation

This study was conducted in two separate locations. The first part, which was conducted in the vivarium at Texas A&M University-Kingsville, involved the induction of Type 1 diabetes in rats using STZ and the subsequent treatment of the diabetic rats with *K. pinnata* aqueous preparation for 30 days post-induction. Traditionally, normal daily dosage of *K. pinnata* aqueous preparation for humans is around three mature leaves (~9.96 g) per 70 kg body weight or about 0.14 grams of plant material / kg body weight. A suitable weight of the plant leaves (the weight of the plant leaves was based on the weight of rats per cage) was homogenized in about 200 milliliters of deionized water daily and poured into sterilized bottles as source of water and plant preparation for the diabetic treated group. The second part of the study was conducted in Dr. Felix Omoruyi's lab at Texas A&M University-Corpus Christi, Natural Resources Center building, room number 3416. Eighteen Sprague-Dawley rats were purchased from Harlan Laboratories Inc. in Chicago, IL. Three groups of rats were used in the study (Table 2), a normal control group receiving deionized water, a STZ-induced diabetic group receiving deionized water, and a STZ-induced diabetic treatment group receiving aqueous preparations of *K. pinnata* daily. Body weight change and total food intake was recorded weekly. The rats were housed in cages with solid flooring covered with a bedding material, and allowed free access to food and liquid for 30 days. On day 31, animals were euthanized by decapitation. Blood samples, intestine, liver, kidneys, and spleen were collected for various assays. Approval for the study was obtained from the Institutional

Animal Care and Use Committee (IACUC) of the Institute of Biosciences and Technology, Texas A&M Health Sciences Center, Houston with protocol number 13001.

Table 2

Summary of Experimental Design

Groups	Diabetes Induction (Day 1)	Diabetes Confirmation (Day 9)	*K. pinnata* Administration (Day 9 - 39)
Group 1	Normal rats administered 0.05M citrate buffer, pH 4.5	Fasting glucose Assay (Control Group)	Normal rats + Deionized water
Group 2	Normal rat diet administered 60 mg of Streptozotocin in 0.05 M citrate buffer, pH 4.5/Kg body weight	Fasting glucose assay for confirmation of diabetes (Diabetic Control Group)	Diabetic rats + Deionized water
Group 3	Normal rats administered 60 mg of Streptozotocin in 0.05 M citrate buffer, pH 4.5/Kg body weight	Fasting glucose assay for confirmation of diabetes (Diabetic Treated Group)	Diabetic rats + *K. pinnata* aqueous preparation

2.2. Objective 1: Determination of glucose utilization, digestive enzymes and intestinal ATPase activities

Induction of diabetes: The diabetic control and diabetic treated groups received a single injection of STZ (Sigma-Aldrich, 60 mg/Kg body weight in 0.05 M-citrate buffer, pH 4.5) intraperitoneally to induce diabetes (Table 2). The normal control group was injected intraperitoneally with an equivalent amount of buffer (0.05 M-citrate buffer, pH 4.5). After 8 days, blood was obtained by pricking the rat tail for a drop of blood for glucose

determination after an overnight fast using a strip-operated blood glucose meter (Bayer Contour Blood Glucose Monitoring System). Diabetes was confirmed when blood glucose was about four times in excess of normal.

Preparation of Intestinal Mucosa Homogenate: The intestine of each rat was divided into two portions: the upper intestine (proximal) and lower intestine (distal). Rat intestine, which was free of food materials, was excised and the lumen was flushed out several times with 0.9% NaCl and the washed mucosa was collected. After washing, the residual mucosa was scraped from the intestine and combined with the washed mucosa, homogenized and centrifuged at 5,000 x g. The supernatant was frozen at -80ºC until required for enzymatic assays [33].

Determination of Intestinal ATPase Activities: Three solutions were prepared for the determination of ATPase activity. Solution A contained 100mM NaCl, 5mM KCl and 6mM $MgCl_2$. Solution B contained 6mM of $MgCl_2$ alone and solution C contained 6mM $MgCl_2$ and 150mM $CaCl_2$. Specific amounts of the membrane samples were added along with 3mM disodium adenosine -5'-triphosphate (ATP) to each solution, the tubes were mixed and the absorbance was measured at 340nm.The activity in solution B was indicative of Mg^{2+} ATPase activity. To determine the level of Na^+/K^+ stimulated activity, the absorbance in solution B was subtracted from the absorbance in solution A. The activity in solution C was indicative of Ca^{2+}/Mg^{2+}ATPase activity. To determine the level of Ca^{2+} ATPase activity, the absorbance in solution C was subtracted from the absorbance in solution B [34–38].

Determination of Intestinal Amylase Activity: Amylase hydrolyzes the α (1-4) linkage of glycogen and starch to yield D-glucose, a small amount of maltose and a resistant core of dextrin [39]. Intestinal mucosa amylase activities were determined using the Stanbio amylase assay kit.

2.3. Objective 2: Blood lipid profile, lipid peroxidation and antioxidant enzyme activity assays

Isolation of Erythrocyte Membrane: In this study, red blood cell (RBC) membranes were prepared from the blood samples collected in EDTA-coated tubes. Briefly, the blood samples were centrifuged at 3,000 x g for 20 minutes at 4°C. The packed cells were washed three times with isotonic 0.3 M Tris-HCl buffer (pH 7.4). An aliquot of 1.0 ml washed cells were lysed using nine milliliters of hypotonic 0.015 M Tris-HCl buffer (pH 7.2). The lysed cells were centrifuged for 30 minutes at 15,000 x g [40]. The pellet was repeatedly washed with hypotonic Tris-HCl buffer until a clear pale pink or colorless supernatant was obtained. The resulting erythrocyte membrane was suspended in 0.01 M Tris-HCL buffer (pH 7.4) and used for the determination of protein and ATPase activities.

Erythrocyte Membrane Protein: Erythrocyte membrane total protein was determined by treating the erythrocyte membrane extract with copper ions in alkaline solution, which formed a colored complex between the copper and the carbonyl and imine groups of protein peptides [41,42]. The violet color that developed (550nm) was proportional to the number of peptide bonds in the protein [41,42]. This procedure was conducted using the Stanbio Total Protein assay kit.

Erythrocyte Membrane ATPases: Erythrocyte membrane ATPase activities were determined using the methods described earlier for intestinal homogenate.

Serum Proteins: The total protein was assayed using the Stanbio total protein kit, as described for erythrocyte membrane protein. Albumin was determined using a bromocresol green (BCG) dye. The methodology was a modification of Dumas, Watson, and Biggs [43,44], where citrate buffer was used instead of a succinate buffer and the absorbance was measured at 550nm rather than 630nm. This procedure was conducted using the Stanbio Albumin Assay kit. The globulin level was calculated by subtracting the albumin level from the total protein [39].

Serum Lipids: Total cholesterol was determined by using cholesterol esterases that hydrolyzed esters to free cholesterol and fatty acids. The free cholesterol was oxidized by Cholesterol oxidase (COx) and combined with 4-aminopenazone in the presence of peroxidase to form a quinoneimine chromogen which was detected spectrophotometrically at 500nm [45,46]. In order to determine HDL cholesterol, the low density lipoproteins (LDL) were precipitated out of the solution using a phosphotungstic acid/magnesium chloride reagent. HDL cholesterol level was determined in the resulting solution using the method for total cholesterol described above [47]. These two assays were performed using the Stanbio Cholesterol kit.

Serum triglycerides: In order to determine triglyceride level, glycerol and fatty acids are first formed by lipase action on the triglycerides. Glycerol is then phosphorylated by ATP to produce glycerol-3-phosphate (G3P) and adenosine-5'-diphosphate (ADP) by glycerol

kinase [48]. G3P is converted through a series of steps to quinoneimine which is then detected at 500nm. This assay was conducted using the Stanbio Triglyceride kit.

Liver and kidney lipid extraction: A known weight of liver or kidney was added to a suitable volume of water and methanol-chloroform (2:1 v/v) mixture and blended for two minutes at room temperature [49]. The residue was re-extracted with a known volume of methanol-chloroform-water (2:1:0.8 v/v/v). After centrifugation, the combined supernatants were diluted with equal volumes each of chloroform and water and the phases separated with a separatory funnel. The chloroform was allowed to evaporate and the sample was used for determination of lipids.

Blood electrolytes: Serum magnesium, phosphorus, chloride, sodium, and potassium concentrations were determined using Stanbio kits. Magnesium was measured by mixing the serum with Xylidyl Blue-1 under alkaline conditions to form a water soluble red purple chelate that absorbs at 520nm [50]. To determine the levels of phosphorus, phosphate ions were mixed with ammonium molybdate, and the product was reduced to form the color pentavalent 'molybdenum blue' and the absorbance was read at 700nm [37,51,52]. Chloride reacts with mercuric thiocyanate to release thiocyanate ions [53,54]. These ions interact with ferric ions to form a reddish ferric thiocyanate, which was detected at 500nm. To determine sodium levels, a protein-free supernatant was precipitated and the sodium was extracted out. The decrease in absorbance of the supernatant color reagent mixture was proportional to the sodium content of the organism [55]. Potassium levels were determined by mixing with a protein-free alkaline medium which reacts with sodium tetraphenylboron to produce finely dispersed turbid suspension

of potassium tetraphenylborate [56]. The turbidity was proportional to the potassium concentration.

Lipid Peroxidation: The formation of lipid peroxides was measured in the serum. The formation of MDA, an end product of fatty acid peroxidation was measured spectrophotometerically at 532 nm by using a thiobarbituric acid reactive substance (TBARS) as described by Genet et al. [57]. The final reaction mixture of three milliliters contained the following: 1.5 ml of 10 mM potassium phosphate buffer (pH 7.4), 0.5 ml of the serum, 0.5 ml of 30% trichloroacetic acid and 0.5 ml of thiobarbituric acid (TBA) (0.53%). The mixture was heated for one hour at 80°C and cooled. The contents were centrifuged at 2,700 x g for five minutes at 4°C and the absorbance was measured in the cleared supernatant at 532 nm against a blank.

Antioxidant Enzymes: The main enzymes that have been implicated in the oxidative degradation of lipids in diabetic patients are Superoxide Dismutase (SOD) and Catalase (CAT). SOD activity was determined using the method of Marklund and Marklund [58] with some modification, by determining the ability of SOD to inhibit the autoxidation of pyrogallol by 50%. The assay mixture of one milliliter contained 500 µl of 0.1 M sodium phosphate buffer, 32 µl of 3.3 mM EDTA, 60 µl of 8.1 mM pyrogallol and appropriate amount of serum containing 7–10 µg of protein. The change in absorbance at 420 nm of the assay mixture was monitored for two minutes at 25°C against a blank that contained all the ingredients except the tissue homogenate. The CAT assay was based on the method by Aeibi [48]. The assay mixture of one milliliter contained 500µl of 0.1M sodium phosphate buffer, 100µl of hydrogen peroxide, and 20µl of homogenate treated with Triton X-100. An equal part of the serum was mixed with Triton X-100 and 20µl of

the resulting homogenate was used for this assay. The decrease in absorbance was then followed at 240nm for five minutes at 25ºC against a blank containing all the ingredients without the enzyme sample. The glutathione (GSH) assay was conducted using Ellman's method [59]. The serum was mixed with 10% trichloroacetic acid (TCA) and centrifuged at 3,000 x g. One milliliter of supernatant was treated with 0.5ml of Ellman's reagent [19.8mg of 5,5'-dithiobisnitro benzoic acid (DNTB) in 100ml of 0.1% 0.2M phosphate buffer (pH 8.0)]. The absorbance was then read at 412nm.

2.4. Objective 3: Determination of Enzymes of Carbohydrate and Lipid Metabolism in the Liver and Kidney

In this study, the possible role of the herbal preparation in reversing the normal metabolic pathways in the liver and kidney was determined by measuring the activities of the following enzymes: glucose-6-phosphatase, $NADP^+$ isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, malic enzyme, and pyruvate kinase. Enzymatic activities were measured using continuous spectrophotometric rate determination methods as outlined below. All assays were conducted at room temperature. The extraction buffer was made up as follows: 60 mM sucrose, 220 mM mannitol, 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, and 5mM dithiothreitol (1:10 w/v). Liver and kidney samples were weighed and homogenized in the extraction buffer above. Homogenates were centrifuged at 5,000 x g for 20 minutes and the supernatant was used as the source of the enzymes.

Pyruvate Kinase: Pyruvate produced by phosphoenolpyruvate was acted upon by lactate dehydrogenase and the oxidation of NADH was measured to assign activity to this

enzyme [60]. The assay mixture contained 0.98 ml of 100 mM Tris-HCl buffer, pH 7.5, 5 mM phenolpyruvate, 5 mM ADP, 0.2 mM NADH, 10 mM $MgCl_2$, 100 mM KCl and excess lactate dehydrogenase (14U). The reaction was started by the addition of tissue homogenate (0.02 ml). The activity was then normalized to the protein concentration in the sample. Protein concentrations were determined using the Stanbio Total Protein assay kit, as previously discussed.

Malic Enzyme: Malic enzyme activity was measured using the method of Storey and Bailey [60]. The assay mixture contained 0.98ml of 100 mM Tris-HCl buffer pH 7.5, 1.0 mM malate, 0.3 mM $NADP^+$ and 0.5 mM $MnCl_2$. The reaction was started by adding 0.02 ml tissue homogenate to the assay mixture in a 3-ml quartz cuvette. Spectrophotometric readings were taken at 340 nm for one minute to detect any endogenous reducing activity. To begin the reaction, 0.1 ml of 0.3 M L-malate was added to the cuvette. Readings were taken after another minute at 340 nm. The change in optical density as $NADP^+$ was converted to NADPH after adding the malate minus the change due to endogenous activity was used for calculation of malic enzyme activity (nanomoles of $NADP^+$ reduced per minute). The activity was then normalized to the protein concentration in the sample.

$NADP^+$*Isocitrate Dehydrogenase: NADP*$^+$-Isocitrate dehydrogenase activity was monitored spectrophotometrically at 340 nm as described by Loftus [61]. One unit of $NADP^+$-dependent isocitrate dehydrogenase activity was defined as the amount of enzyme required to catalyze the oxidative decarboxylation of 1.0 µmol of D-isocitrate to α-ketoglutarate in one minute under standard assay conditions. The assay mixture contained 0.98ml of 100 mM Tris-HCl buffer, pH 8.0, 0.8 mM isocitrate, 0.4 mM

$NADP^+$ and 2 mM $MgCl_2$. The reaction was started by the addition of 0.02ml tissue homogenate. The activity was then normalized to the protein concentration in the sample.

Glucose 6-phosphate Dehydrogenase: The assay mixture contained 0.98ml of 100 mM Tris-HCl buffer, pH 8.0, 1.0 mM glucose-6-phosphate, 0.5 mM $NADP^+$ and 5.0 mM $MgCl_2$. The reaction was started by the addition of 0.02 ml tissue homogenate. It was mixed immediately by inversion and the increase in absorbance at 340nm was recorded for approximately five minutes [60].

Liver and Kidney Function Enzymes: Blood urea nitrogen (BUN), bilirubin, creatinine and uric acid were assayed to determine if renal damage occurred due to treatment; additionally, alanine and aspartate amino transferases (ALT and AST), and ALP were assayed to determine if hepatic dysfunction was a side effect of the aqueous preparation consumption. In order to determine BUN level, acid catalyzed condensation of urea reacted with diacetylmonoxime in the presence of thiosemicarbazide to produce a red-purple chromogen which was measured spectrophotometrically at 520nm [62]. Creatinine levels were determined by mixing the serum with picric acid in alkaline conditions to produce a color complex that was measured spectrophotometrically at 510nm. While dry picric acid is highly sensitive to shock and friction, the picric acid used in lab was contained in an aqueous solution. However since the compound may still be highly unstable, the assay was conducted carefully under the hood with eye shields and gloves. After the assay was completed, the solution was poured into a glass bottle, labeled properly, and disposed in the biohazard bin. The rate of color formation was proportional to the creatinine concentration [63]. Bilirubin was determined through a series of steps where p-benzenediazoniumsulfonate couples with bilirubin to produce azobilirubin,

which was detected at 540nm [64]. Uric acid was broken down by uricase to form hydrogen peroxide and allantoin. Hydrogen peroxide was measured quantitatively by its reaction with 3,5-dichloro-2hydroxybenzenesulfonic acid (DCHB), in the presence of peroxidase and 4-aminophenazone to form a red violet quinoneimine complex [65,66]. This color change was analyzed at 520nm and was proportional to the amount of uric acid in the system. ALT and AST levels were determined based on the oxidation of NADH by lactate dehydrogenase (LDH). NADH was absorbed at 340nm and the rate of decrease was directly proportional to ALT activity [67,68]. ALP was measured using the hydrolysis of the substrate 4-nitrophenyl phosphate to form 4-nitrophenol and phosphate, which was measured at 405nm [69]. The rate of 4-nitrophenol production was directly proportional to the ALP activity. AST catalyzes the transfer of the amino group aspartate to α-ketoglutarate to yield oxaloacetate and glutamate [70]. Glutamate then reacts with NADH in the presence of malate dehydrogenase to form NAD. The rate of oxidation of NADH was measured at 340nm. These parameters were measured using Stanbio kits.

2.5 Objective 4: Determination of complete blood count and inflammatory responses

White Blood Cell Count: The white blood cell levels were determined by performing a Complete Blood Count (CBC) using a Siemens Advia 120 systems analyzer. The samples were sent out to a local hospital laboratory for analysis.

Determination of Hemoglobin Content: Hemoglobin content in the blood was determined in the CBC report as well.

Cytokine Determination in the Blood: A relative balance in the cytokine environment is critical for maintaining protective immunity and avoiding immune injury [33]. This study

was conducted to determine if the herbal preparation induced changes in cytokine [interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)] contents in the blood. The serum was assayed for the contents of IL-1β, IL-6 and TNF-α using ELISA kits (Fisher Scientific, IL, USA).

2.6 Statistical Analysis

Results are presented as means ± SEM (n = 6). Analysis of variance (ANOVA) was used to test differences between the groups. Duncan's Multiple Range Test at the significance level of $P < 0.05$ was used to test the significance among the means.

3. RESULTS and DISCUSSION

3.1. Body and Organ Weight Changes and Food Intake

During the 30 day study the changes in body weight and amount of food consumed by each animal were measured. The diabetic treated group consumed similar amounts of food, but lost the most weight compared to the other two groups (Figure 4). Gwon et al. [71] reported that the inhibitory effect of *Zanthoxylum piperitum (Z. piperitum)* extract (ZPE) on high fat diet-induced obesity may be partially attributed to flavones, which could also explain the decrease in body weight in our study. The leaves of *K. pinnata* are known to contain flavonoids, polyphenols, triterpenoids and phytosterols [72]. In addition, several systemic peptides have been shown to regulate appetite and body weight. We suggest further studies on the potential role of *K. pinnata* aqueous preparation on vital systemic peptides involved in the regulation of appetite and body weight. We also noted a non-significant decrease in liver and kidney weights in the treatment group compared to the other groups (Figure 5).

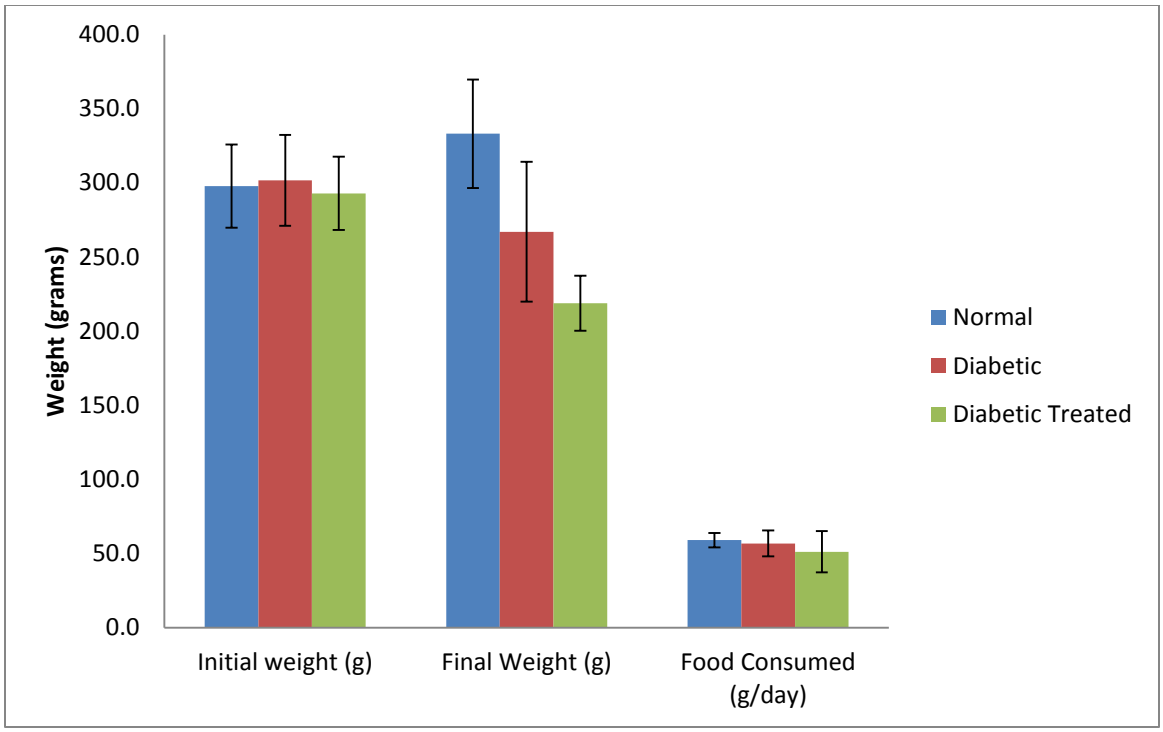

Fig. 4 Body Weight Changes and Food Intake of Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata*. Values were not significantly different among the groups ($P > 0.05$)

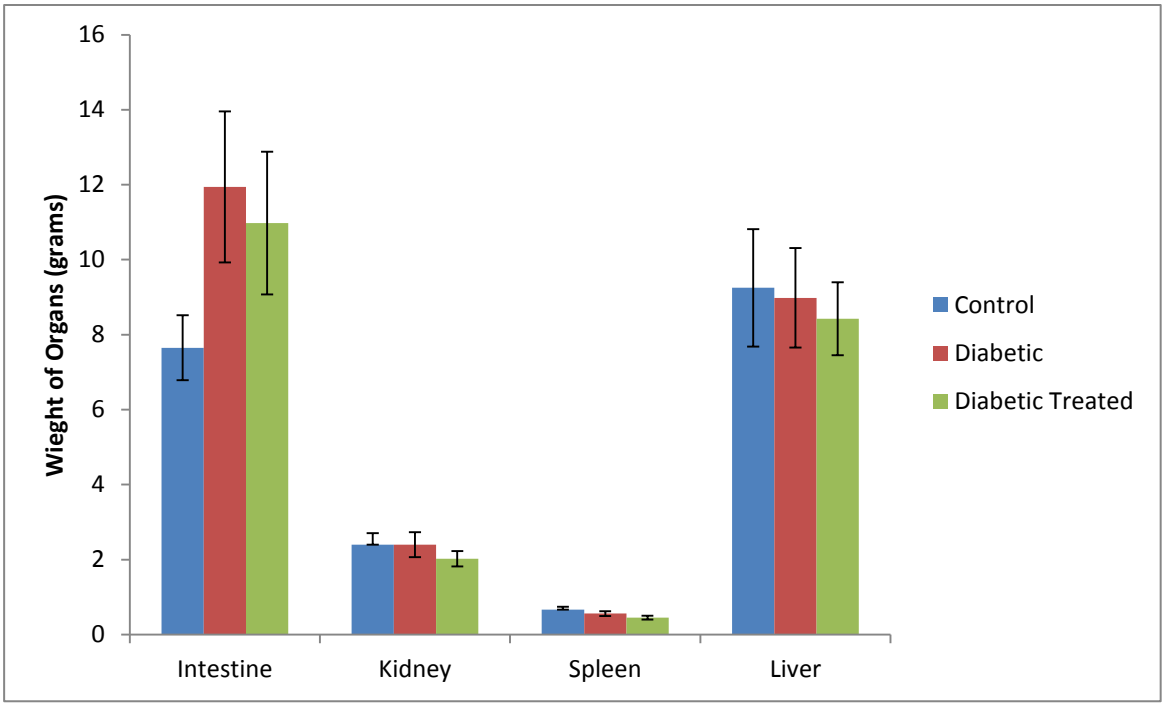

Fig. 5 Average Organ Weight of Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata.* Values were not significantly different among the groups ($P > 0.05$)

3.2. Serum Glucose and Proteins

Hyperglycemia is a characteristic feature of diabetes [73]. In this study, we noted a significant ($P < 0.05$) decrease in serum glucose level (Figure 6) in the diabetic treated group compared to the diabetic control group. This is supported by previous studies that have also identified *K. pinnata* as a potent hypoglycemic agent [29,74]. The chemical constituents of the herb are believed to account for the observed hypoglycemic property of the plant [72]. The presence of zinc in the plant may also play a role in its hypoglycemic activity. Zinc is able to exert insulin-like effects by supporting the signal transduction of insulin and reducing the production of cytokines that contributes to beta-cell death in the pancreas [75]. Additionally, in this study we also noted a significant ($P < 0.05$) increase in hepatic pyruvate kinase activity (Figure 14) in the diabetic treated group. Previous studies have noted that treatment with insulin restores pyruvate kinase activity and recovers diminished glycolytic activities in untreated diabetes [76].

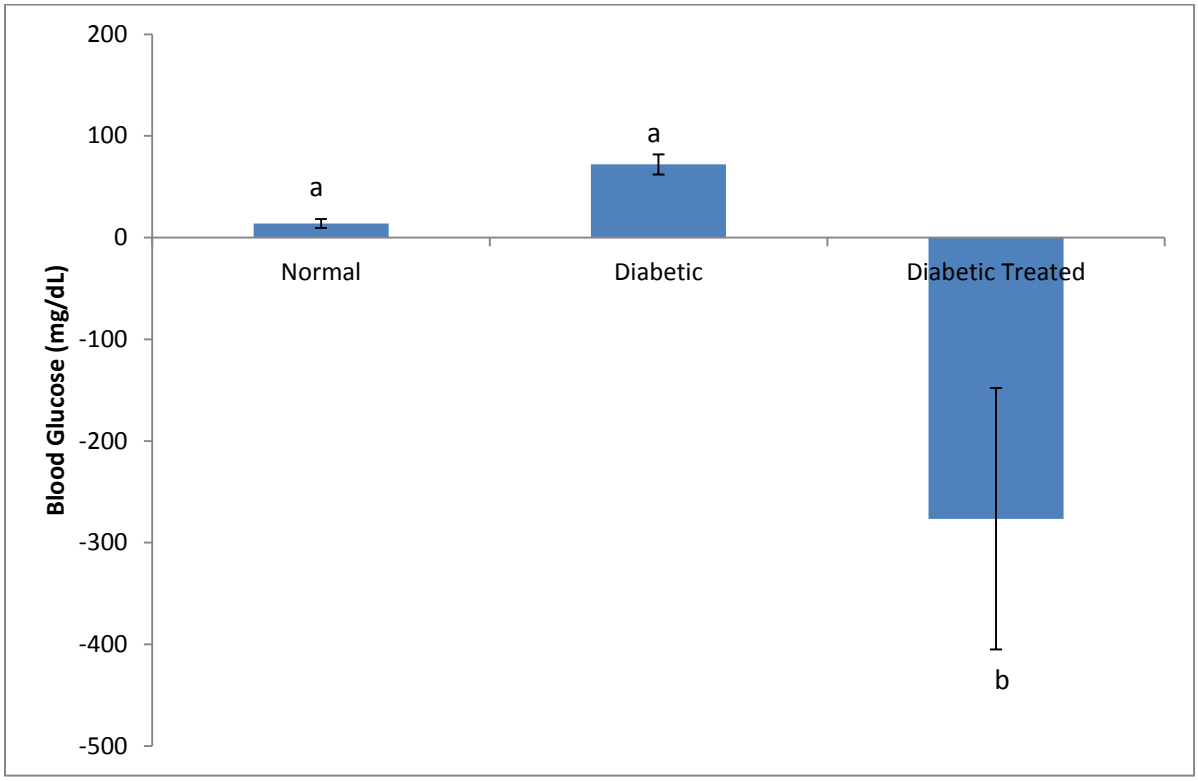

Fig. 6 Change in Blood Glucose in Diabetic Rats Administered Aqueous preparation of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$)

Elevated levels of plasma glucose modify proteins by a non-enzymatic reaction called glycation. Protein glycation leads to the formation of heterogeneous fluorescent molecules called advanced glycation end products (AGEs) [77]. Accumulation of AGEs has been found to be accelerated in diabetes and to contribute to the pathogenesis of diabetic complications. In this study, Serum albumin level was significantly ($P < 0.05$) reduced in the diabetic control group and there was a non-significant increase in the diabetic treated group towards the normal control group (Figure 7a). Low levels of albumin are associated with increased plasma protein glycation and increased levels of glycated hemoglobin [77]. Glycated albumin has been associated with the high prevalence of macrovascular disease in diabetes mellitus [78]. Furthermore, AGEs may interact with receptors for AGEs (RAGE), triggering a cascade of events leading to oxidative stress and proinflammatory pathways [77]. Thus, the observed increasing but non-significant trend in blood albumin level in the diabetic treated group may indicate a reduction in endothelial dysfunction and protein glycation. However, the effect of prolonged consumption of aqueous preparation of *K. pinnata* on serum albumin level in diabetes needs further investigation.

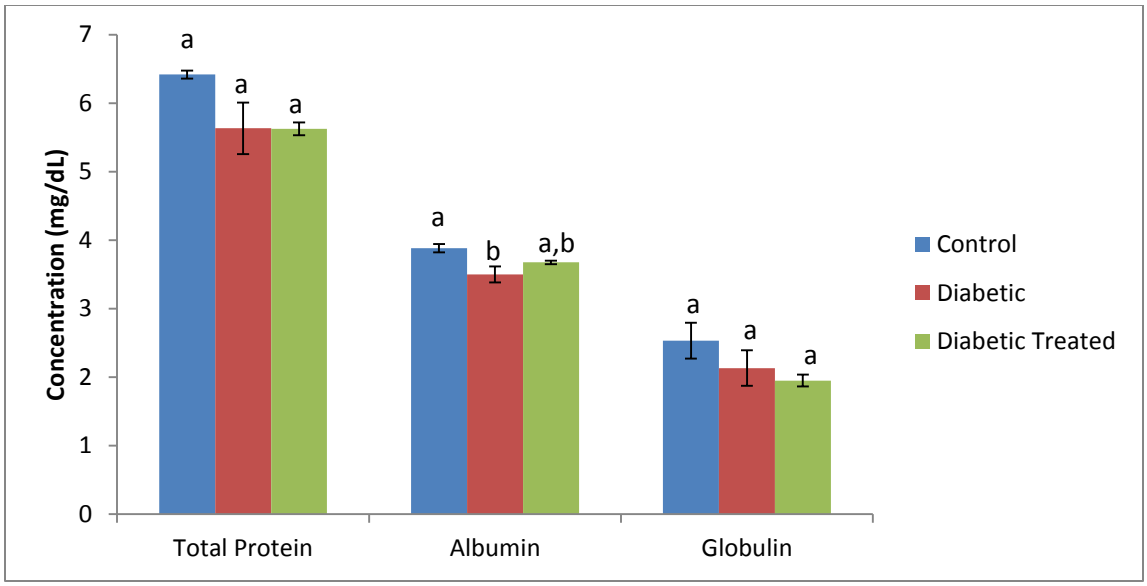

Fig.7a Change in Serum Protein Profile in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$)

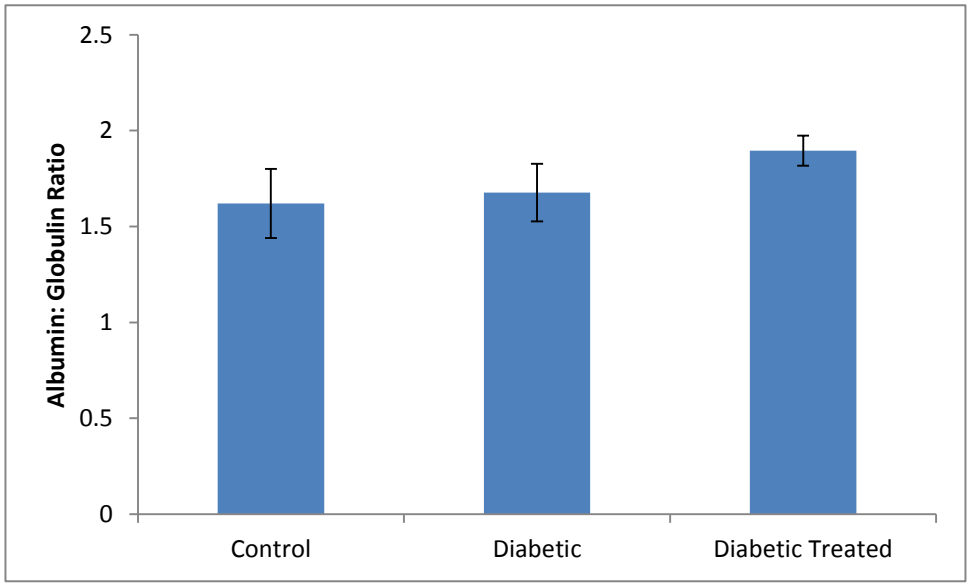

Fig.7b Albumin:globulin ratio in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata.* Values were not significantly different among the groups ($P > 0.05$)

3.3. Serum Lipid Profile

Earlier reports have shown that diabetes is strongly associated with cardiovascular disease [79–81]. Diabetic dyslipidemia is characterized by moderate elevations in triglyceride levels, low HDL cholesterol values, and small dense LDL particles [79]. In this study, we noted a significant ($P < 0.05$) elevation in serum triglyceride level in the diabetic control group, which was reduced towards normal level by the treatment (Figure 8). Serum total cholesterol level was also elevated in the diabetic control group and there was a decreasing trend towards the normal control group by the treatment (Figure 8). Additionally, serum HDL cholesterol was significantly ($P < 0.05$) reduced in both the diabetic control and diabetic treatment group (Figure 8) compared to the normal control group. Elevated triglyceride level and reduced HDL levels are conducive to the development of atherosclerosis and increases the risk of cardiovascular disease [82]. In the diabetic state, there is a defect in the metabolism of stored triglycerides by adipose tissue, resulting in elevated transport of free fatty acids (FFA) to the liver, which triggers the overproduction of large VLDL fragments and triglycerides [21]. This results in secondary abnormalities of low HDL-cholesterol and increased LDL particle and density [83]. Elevated level of LDL cholesterol is subject to oxidative modification and transformation into foam cells, which accumulate in the form of fatty streaks and fibrofatty plaques that lead to the development of atherosclerosis [21]. Although the treatment was able to decrease the triglyceride level which may reduce cardiovascular risk, the non-restoration of HDL-cholesterol level towards the normal control group may be a drawback in the use of the preparation for the management of diabetes. However, the evaluation of the entire lipid profile which includes LDL, VLDL, and lipid moieties is

required to ascertain whether the tissues are taking up excess lipids or improving fuel utilization.

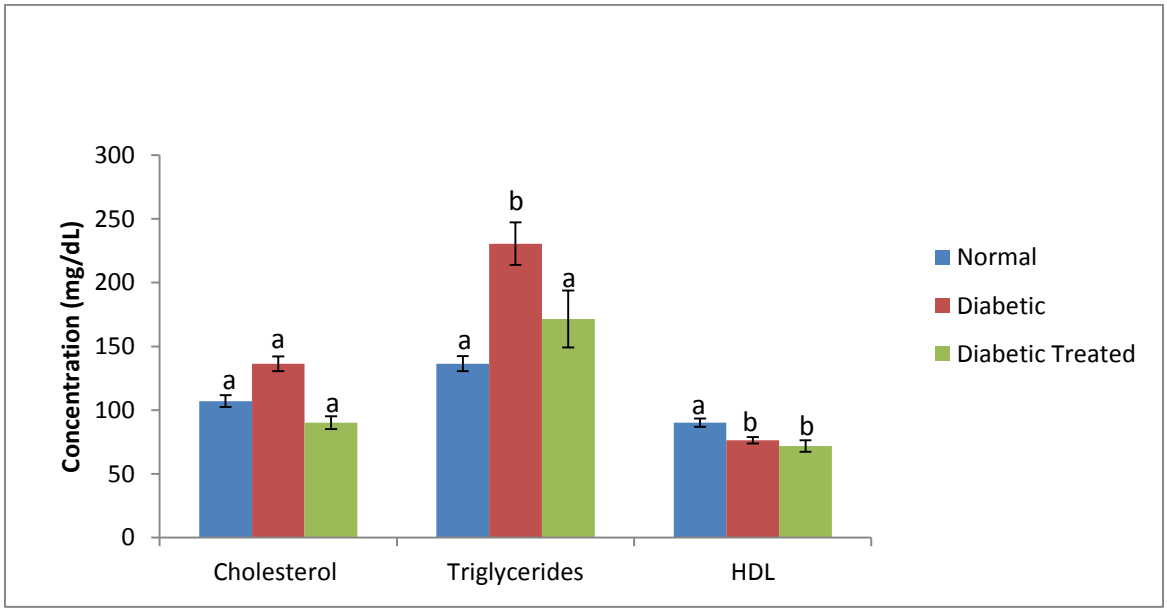

Fig. 8 Change in Serum Lipid Profile in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$)

Diabetes mellitus is known to be associated with a number of liver disorders, including nonalcoholic fatty liver disease (NAFLD), hepatitis C infection (HCV), cirrhosis and hepatocellular carcinoma [84–86]. It is thought that decreased insulin sensitivity, a cornerstone in the pathogenesis of type 2 diabetes (T2D), activates lipolysis leading to increased plasma levels of non-esterified fatty acids. A resultant chronic increase in fatty acid flux from the fat stores (mainly abdominal) to non-adipose tissues such as the liver, contributes to hepatic steatosis [87,88]. The liver is a major insulin-sensitive metabolic organ that controls glucose and lipid homeostasis [81]. We noted an increasing trend in the liver triglyceride level in the diabetic control group compared to the normal control group (Table 3). Treatment with *K. pinnata* preparation showed a tendency to decrease the level of liver triglyceride towards normal control group (Table 3). The increased rate of hepatic triglyceride synthesis and VLDL production in diabetes result in secondary abnormalities of low HDL and increased LDL particle number and density [83]. Insulin resistance is regarded as a major driving force for dyslipidemia, with one mechanism being an increase in free fatty acid [80,89–92]. Elevated levels of triglycerides and FFA lead to the development of fibrofatty plaques and the development of cardiovascular disease. The observed decreasing trend in liver triglyceride level would suggest that the administration of *K. pinnata* preparation may be beneficial in the management of diabetes.

Table 3
Effect of Aqueous *Kalanchoe pinnata* Preparation on Liver Lipid Profile

	Cholesterol (mg/g tissue wet weight)	HDL (mg/g tissue wet weight)	Triglycerides (mg/g tissue wet weight)
Normal Control	0.65 ± 0.16	0.36 ± 0.07	4.09 ± 1.05
Diabetic Control	0.76 ± 0.13	0.36 ± 0.06	4.46 ± 0.93
Diabetic Treatment	0.71 ± 0.23	0.32 ± 0.13	3.01 ± 1.75

Values were not significantly different among the groups ($P > 0.05$)

Our data also showed significant increase in total cholesterol level in the kidney ($P < 0.05$) in the diabetic control group compared to the other groups. There was a significant ($P < 0.05$) decrease in total cholesterol level in the diabetic treated group compared to the diabetic control group (Table 4). Treatment with *K. pinnata* preparation produced a decreasing but non-significant trend in kidney HDL-cholesterol and triglyceride levels toward that of the normal control group (Table 4). There is growing evidence that abnormalities in lipid metabolism contribute to renal disease progression. Elevated levels of lipids in the kidney are associated with an increased rate of glomerulosclerosis (hardening of the glomeruli in the kidney), and reductions in lipid levels reverse this effect [79]. The observed decrease in kidney lipids in the diabetic treated group may prevent the development of glomerulosclerosis. This may be indicative of the beneficial effect of *K. pinnata* preparation consumption in the management of diabetes.

Table 4

Effect of Aqueous *Kalanchoe pinnata* Preparation on Kidney Lipid Profile

	Cholesterol (mg lipid/g tissue wet weight)	HDL (mg lipid/g tissue wet weight)	Triglycerides (mg lipid/g tissue wet weight)
Normal Control	2.72 ± 0.68^{a}	0.22 ± 0.06^{a}	4.93 ± 0.96^{a}
Diabetic Control	7.98 ± 1.61^{b}	0.37 ± 0.09^{a}	4.27 ± 1.73^{a}
Diabetic Treated	2.47 ± 0.67^{a}	0.26 ± 0.08^{a}	1.63 ± 0.28^{a}

Figures that share different letter superscripts are significantly different ($P < 0.05$)

3.4. Oxidative Stress

Elevated levels of plasma glucose have been implicated in glucose autoxidation, non-enzymatic protein glycation, and activation of polyol pathway with increased oxidative stress. Hyperglycemia in particular has been found to promote lipid peroxidation of low density lipoprotein, that results in the generation of free radicals [93]. In this study, we noted decreased serum SOD and CAT activities, and GSH levels in the diabetic control group compared to the other groups, but most measures were non-significant (Table 5). Catalase activity was significantly ($p < 0.05$) increased in the diabetic treated group compared to the other groups (Table 5). Antioxidant enzymes are responsible for rapidly and efficiently scavenging free radicals and converting them to non-toxic products in order to prevent cellular damage. High levels of free radicals can cause damage to cellular proteins, membrane lipids, nucleic acids and subsequently cell death [93]. The increase in antioxidant indices in the diabetic treated group may be indicative of an improved antioxidant defense system.

Table 5
Alteration of Antioxidant Indices in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata*

	SOD (U/mg protein/min)	GSH (uM/mg protein)	CAT (mmol H_2O_2/min/mg protein)	TBARS (mmol MDA formed/mg protein)
Normal Control	0.87 ± 0.18 [a]	202.29 ± 24.63 [a]	0.17 ± 0.04[a]	3.42 ± 0.20 [a]
Diabetic Control	0.61 ± 0.09 [a]	162.03 ± 24.67 [a]	0.13 ± 0.02[a]	3.66 ± 0.33 [a]
Diabetic Treated	0.82 ± 0.21 [a]	208.40 ± 6.93 [a]	0.34± 0.12[b]	3.60± 0.23 [a]

SOD= Superoxide dismutase, GSH = Glutathione, CAT= Catalase, TBARS= Thiobarbituric Acid Reactive Substances

Figures that share different letter superscripts are significantly different ($P < 0.05$)

3.5. Serum and Intestinal Amylase

Low serum amylase is associated with insulin deficiency in patients with Type I diabetes and, less commonly seen in type 2 diabetes [94–97]. Data from this study showed a significant ($P < 0.05$) reduction in serum and intestinal mucosa amylase activity in the diabetic control and diabetic treated groups compared to the normal control group (Figures 9, 10a and 10b). However, we noted a non-significant increasing trend in serum amylase activity in the diabetic treated group compared to the diabetic control group (Figure 9). In most diets, carbohydrates represent the greatest source of calories. During digestion of carbohydrates, insulin binds to its receptor on acinar cells and stimulates amylase secretion via various pathways [98]. The observed increase in serum amylase activity in this study suggests that the consumption of aqueous *K. pinnata* preparation may have a stimulating effect on pancreatic acinar cells such as insulin, and trigger the release of amylase from the pancreas. According to Nakajima et al. [98], low serum amylase may reflect metabolic abnormalities, and the reversal of this trend could indicate a restoration in metabolic activities as observed in the diabetic treated group. Amylase and glucosidase are the main enzymes involved in the breakdown of starch into disaccharides and oligosaccharides into simple sugars such as glucose which are later absorbed from small intestine into the blood circulation [99].

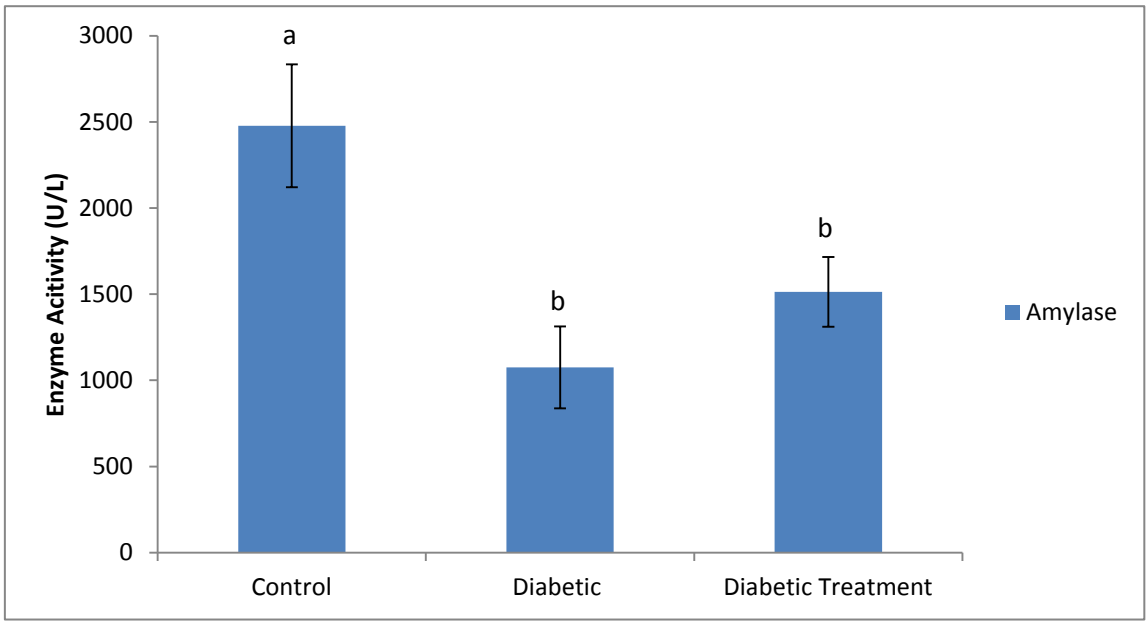

Fig. 9 Amylase Activity in the Serum of Diabetic Rats Administered Aqueous preparations of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$)

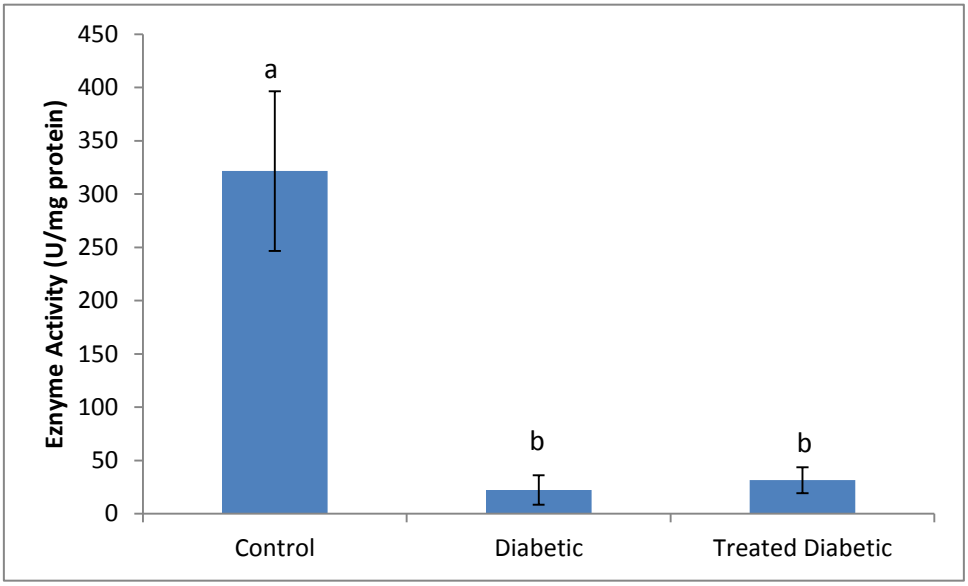

Fig. 10a Amylase Activity in the Proximal Intestinal Mucosa of Diabetic Rats Administered Aqueous Preparations of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$)

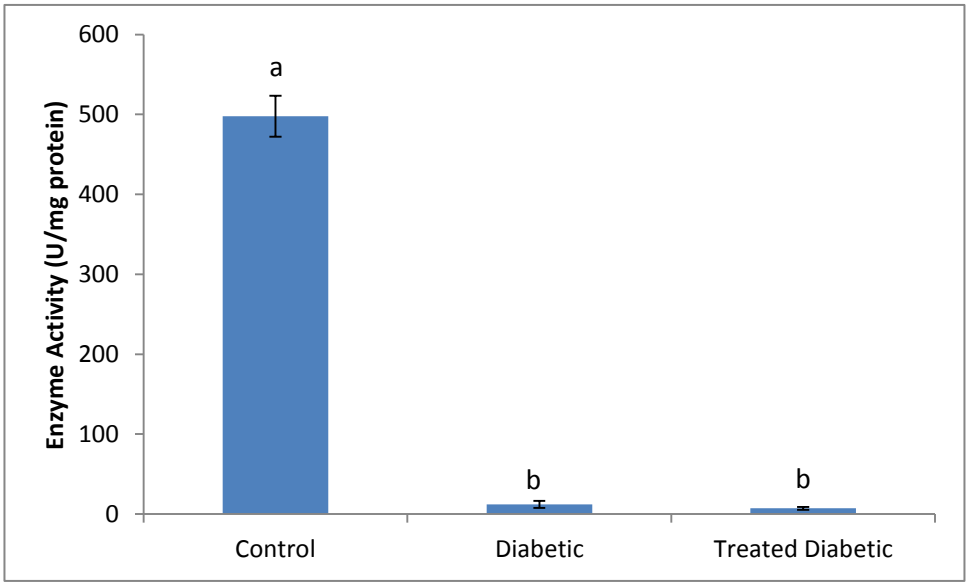

Fig. 10b Amylase Activity in the Distal Intestinal Mucosa of Diabetic Rats Administered Aqueous Preparations of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$)

3.6. Intestinal Mucosa ATPases

Alterations of intestinal mucosa ATPases may affect solute transport and energy production. Ca^{2+} ATPase activity was also slightly reduced in the diabetic treated group compared to the other groups. The activity of Mg^{2+} ATPase was similar among the groups (Figure 11). Barada et al. [100] reported that a possible physiologic role of the increased Na^{+}/K^{+} ATPase activity in diabetes in the small intestine, liver and kidneys is to facilitate an increase in Na^{+}-dependent solute transport. Conversely, it has been proposed that since the subunits of the Na^{+}/K^{+} ATPase exist as multiple isoforms (α1, α2, α3, β1, β2) then diabetic impairment of Na^{+}/K^{+} ATPase activity could be due to alterations in the enzyme activity and/or subunit expression [101]. In this study, there were no significant changes in intestinal Na^{+}/K^{+} ATPase activity among the groups (Figures 11 and 12).

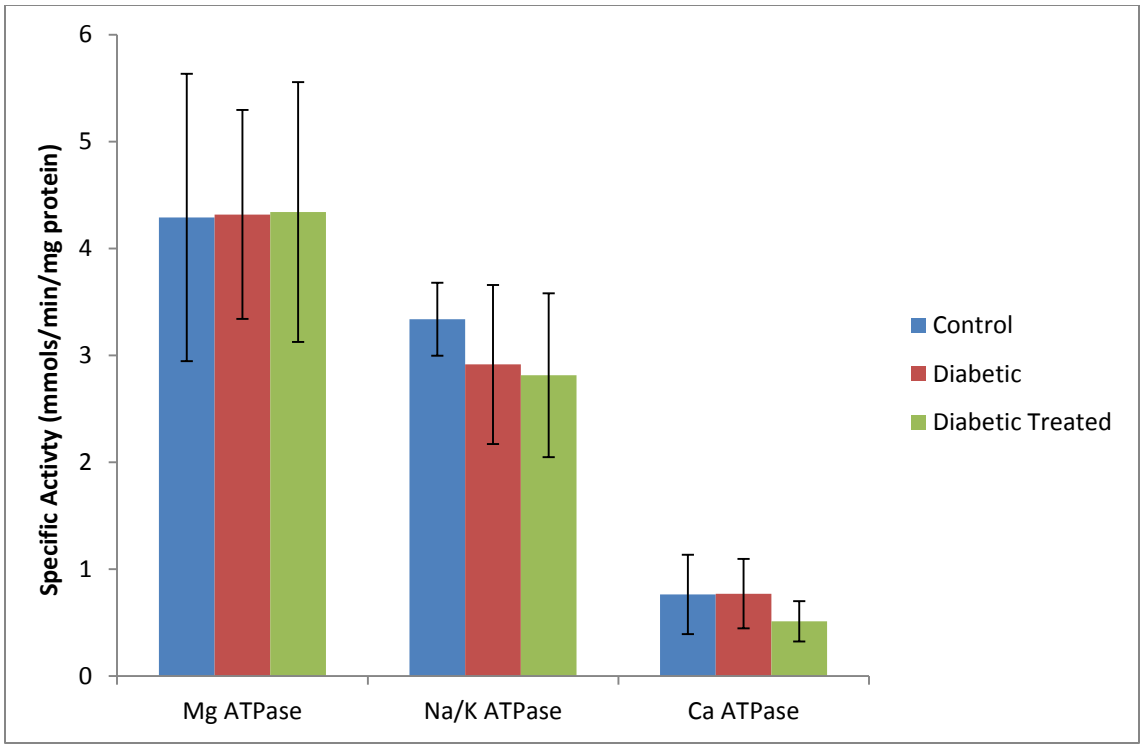

Fig. 11 Proximal Intestinal Mucosa ATPase Activities in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata*. Values were not significantly different among the groups ($P > 0.05$)

However, we noted a significant ($P < 0.05$) increase in Mg^{2+}ATPase activity in the distal region of the gut of diabetic treated group compared to the diabetic control group (Figure 12). Intracellular Mg^{2+} is a critical cofactor for several enzymes in carbohydrate metabolism because of its role as part of the activated Mg^{2+}-ATP complex. It is required for all of the rate limiting enzymes of glycolysis, and the regulation of the activity of all enzymes involved in phosphorylation reactions [102]. Mg^{2+} deficiency may also result in disorders of tyrosine-kinase activity on the insulin receptor, leading to the development of post-receptorial insulin resistance and decreased cellular glucose utilization [103]. The observed increase in distal intestinal Mg^{2+} ATPase activity may suggest that the consumption of aqueous *K. pinnata* preparation could increase intracellular magnesium and subsequently increase the metabolism of glucose via the glycolytic pathway.

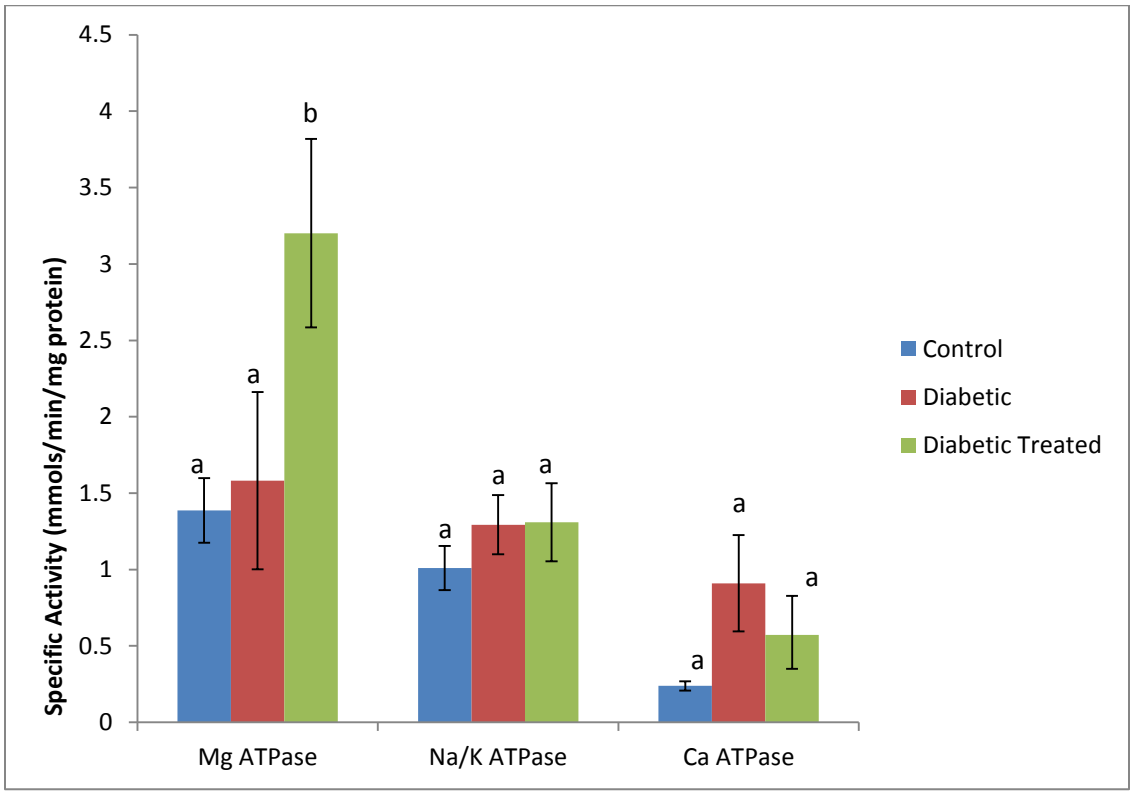

Fig. 12 Distal Intestinal Mucosa ATPase Activities in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata*. Figures that share different letter superscripts are significantly different ($P < 0.05$)

3.7. Erythrocyte Membrane ATPases

Abnormalities in blood viscosity and erythrocyte deformability have been identified as crucial factors in the development of diabetic vascular and microvascular disease. Erythrocyte membrane stability is maintained by unidirectional transport of Ca^{2+} and Na^{+} ions out of the cell, and K^{+} influx into the cell [104]. Diabetes-induced decrease in Na^{+}/K^{+} ATPase activity compromises microvascular blood flow via two mechanisms; by affecting microvascular regulation and decreasing red blood cell deformability, which leads to an increased blood viscosity [105]. Maintenance of the cation gradient by Na^{+}/K^{+} and Ca^{2+}ATPases is essential in the control of hydration, volume, nutrient uptake, and fluidity of cells, and is also essential for the contractility and excitability properties of muscle and nerve tissues [106–108]. In this study Na^{+}/K^{+} ATPase activity was not significantly altered in the erythrocyte membrane of the diabetic treated group compared to the diabetic control group. Ca^{2+} ATPase activity was elevated but not significantly different in both diabetic treated and diabetic control groups compared to the normal control group. We also noted a significant ($P < 0.05$) increase in magnesium ATPase activity in the diabetic treated group compared to the diabetic control group (Figure 13). Increased Mg^{2+} ATPase activity in the erythrocyte membrane acts to reduce cellular calcium content, improve erythrocyte flexibility and reduces vascular complications [109]. The observed increase in Mg^{2+} ATPase activity in the erythrocyte membrane suggests that the consumption of aqueous *K. pinnata* preparation in diabetes may improve erythrocyte membrane flexibility and lower the risk of vascular diseases that are often associated with diabetes mellitus complications.

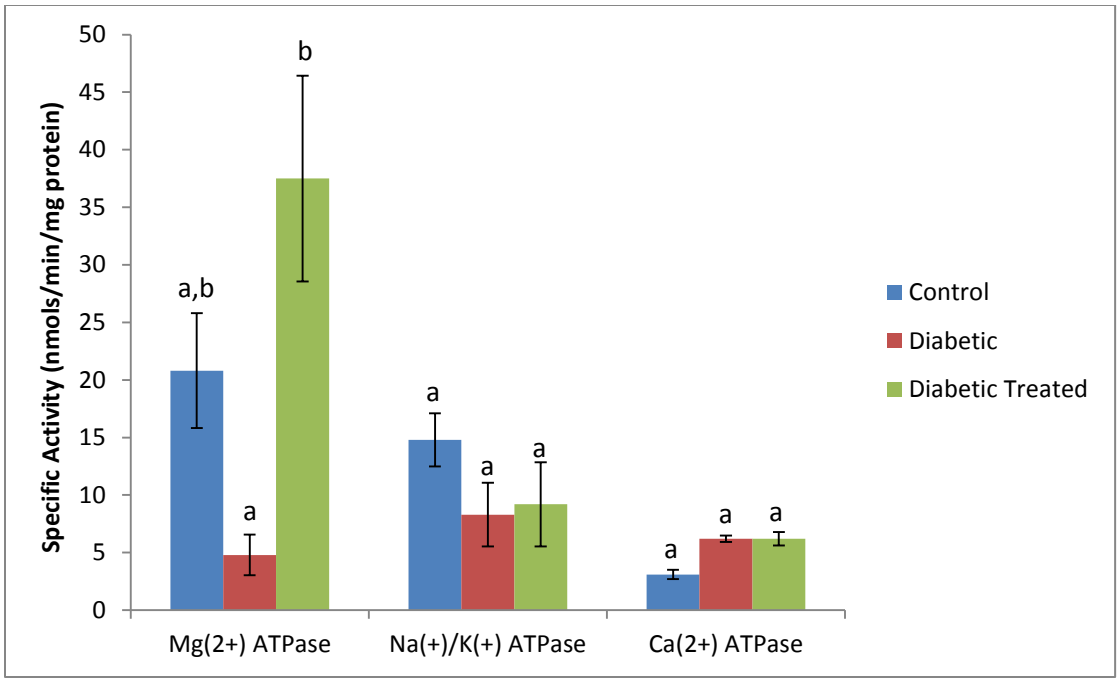

Fig. 13 Erythrocyte Membrane ATPase Activities in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata*. Figures that share different letter superscripts are significantly different ($P < 0.05$)

3.8. Blood Electrolytes

Blood electrolytes are required to maintain the cellular membrane potential, and as a result, they are essential in regulating cellular metabolism and energy transfer. In this study, serum magnesium, calcium, chloride, sodium and phosphorus levels were not significantly altered among the groups (Table 6).

Table 6.
Serum Electrolytes in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata*

	Chloride (mg/dL)	Magnesium (mEq/L)	Sodium (mmol/L)	Phosphorus (mg/dL)	Calcium (mg/dL)
Normal Control	92.38 ±2.71	1.77 ±0.05	74.05 ±17.07	8.99 ± 0.47	11.94 ±1.34
Diabetic Control	102.60 ± 2.99	1.64 ±0.04	99.97 ± 8.27	8.73 ±.036	11.92 ± 0.50
Diabetic Treated	96.40 ± 2.79	1.83 ±0.11	80.02 ± 8.44	8.27 ± 0.59	12.04 ±1.67

Values were not significantly different among the groups ($P > 0.05$)

3.9. Metabolic Enzyme Activities

The liver is the main organ responsible for carbohydrate metabolism. However, both organs (liver and kidney) contain enzymes responsible for glycogenolysis, glycogenesis, and gluconeogenesis. In this study, there were no significant changes in liver malic enzyme, glucose-6-phosphate dehydrogenase and $NADP^+$-isocitrate dehydrogenase activities among the groups (Figure 14). Pyruvate kinase was significantly ($P < 0.05$) increased in the diabetic treated group compared to the diabetic control (Figure 13). Pyruvate kinase is a glycolytic enzyme responsible for the production of pyruvate, the end product of glycolysis [110]. Insulin and other anti-diabetic remedies have been noted to up-regulate the production of pyruvate kinase, facilitating the utilization of intracellular glucose for energy generation [111].

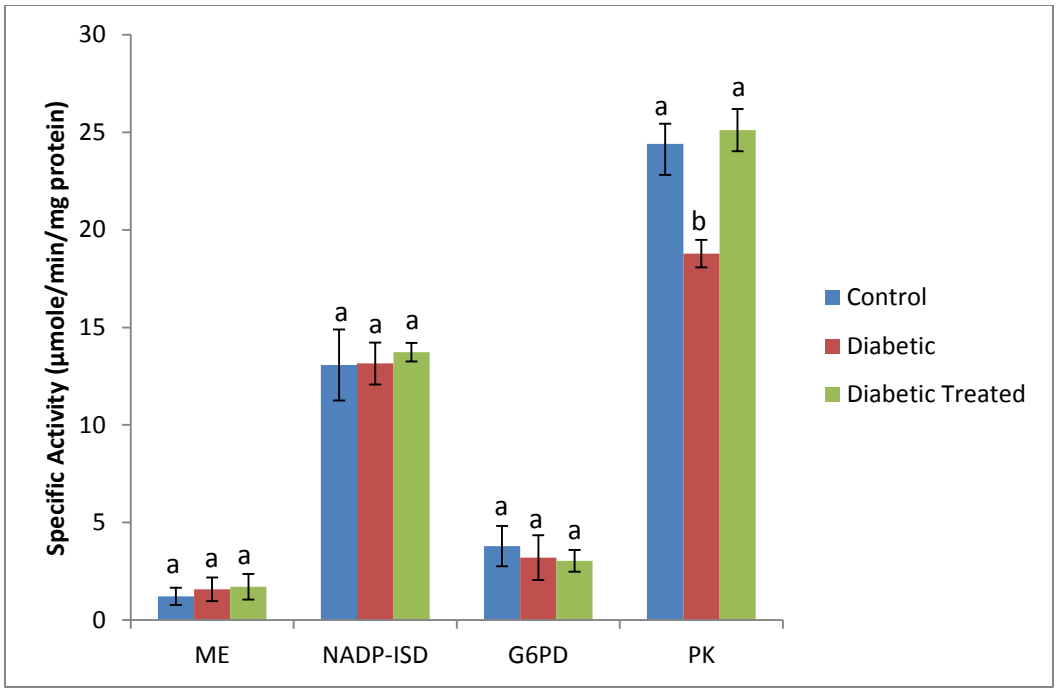

Fig. 14 Metabolic Enzyme Activities in the Liver of Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$). G6PD = Glucose-6-Phosphate Dehydrogenase, ME = Malic Enzyme, NADP-ISD = $NADP^+$-Isocitrate Dehydrogenase, PK= Pyruvate Kinase

The kidney contributes to hyperglycemia through two different mechanisms: enhanced glucose reabsorption and gluconeogenesis [112,113]. Whereas the former is insulin-independent, the latter is negatively regulated by insulin [114]. In this study, we noted a decreasing trend in malic enzyme, glucose-6-phoshate dehydrogenase and pyruvate kinase activities in the kidney of the diabetic treated group compared to the diabetic control (Figure 15). The observed decrease in pyruvate kinase activity is indicative of reduced glycolytic pathway in the kidney of the diabetic treated group. Although the observed decrease in malic enzyme and glucose-6-phoshate dehydrogenase activities may be indicative of reduced demand for NADPH for reductive biosynthesis in the kidney, the observed significant ($P < 0.05$) increase in NADP+-isocitrate dehydrogenase activity in the kidney of the diabetic treated group compared to the other groups may be indicative of a compensatory kidney response geared towards producing NADPH for reductive biosynthesis. This is indicative of an alternate pathway for NADPH production.

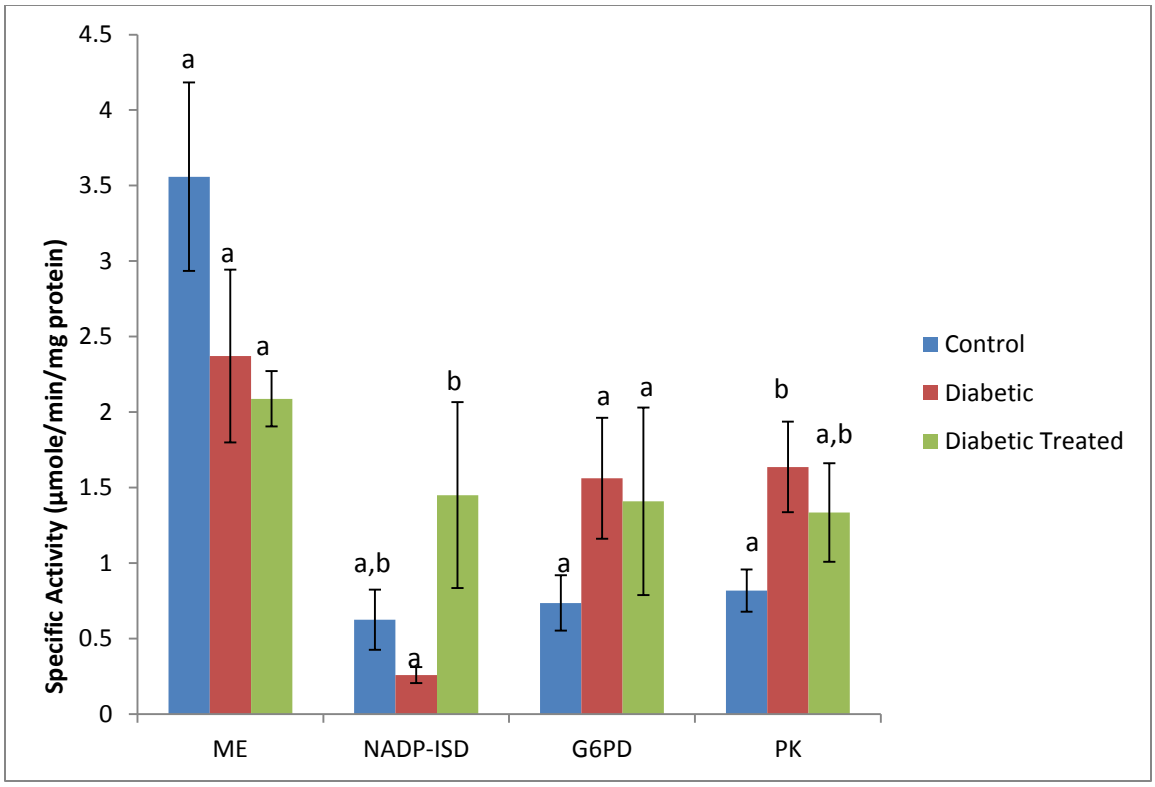

Fig. 15 Metabolic Enzyme Activities in the Kidney of Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$). G6PD = Glucose-6-Phosphate Dehydrogenase, ME = Malic Enzyme, NADP-ISD = $NADP^+$-Isocitrate Dehydrogenase, PK= Pyruvate Kinase

3.10. Organ Function Enzymes

Elevated levels of aminotransferases, such as ALT and AST in the serum may be indicative of hepatocellular injury with subsequent leakage of hepatic enzymes into the circulation. Elevated activities of ALT and AST have been reported in liver disease, obesity, diabetes and dyslipidemia [115]. In this study, we noted a non-significant decrease in blood ALT and AST activities in the diabetic treated group compared to the diabetic control group (Figure 16). However, the observed significant ($P < 0.05$) increase in the ALP activity in both the diabetic control and diabetic treated groups compared to the normal control group may be indicative of damage to other organs. While ALT and AST are specific markers for hepatic damage, ALP activity may be elevated in a variety of diseases such as bone disease, congestive heart failure, liver damage and bile duct problems [115]. Further studies are necessary to evaluate the effect of aqueous *K. pinnata* preparation consumption on these other organs that are also crucial in ascertaining the effective and safe usage of the preparation in the management of diabetes mellitus.

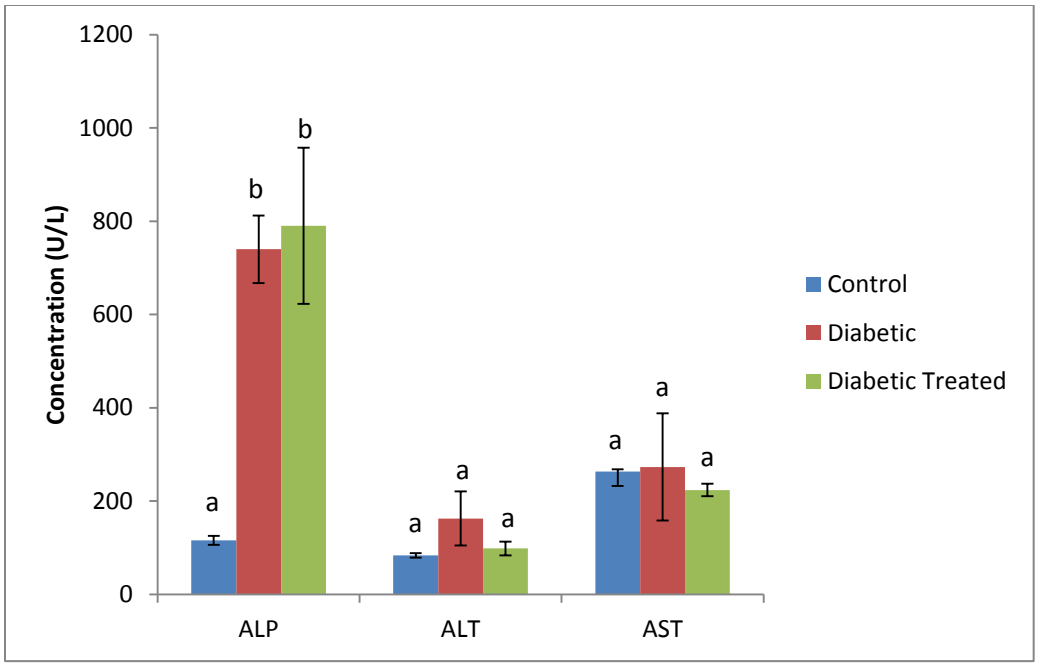

Fig. 16 Liver Function Enzymes in the Serum of Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$). ALP = Alkaline Phosphatase, ALT = Alanine Amino Transferase, AST = Aspartate Amino transferase

Diabetic nephropathy (DN), a frequent and major microvascular complication of diabetes mellitus, is the most common cause of end-stage renal failure disease in many countries around the world [116]. Several factors including hyperglycemia, hyperlipidemia, oxidative stress and inflammatory cytokines, contribute to the progression of renal damage in diabetic nephropathy [116]. In this study, the levels of serum creatinine and uric acid were not significantly altered among the groups (Table 7). However, the observed significant ($P < 0.05$) increase in BUN in the diabetic control and diabetic treated groups may be unrelated to renal function [117].

Table 7.
Kidney Function Enzymes in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata*

	Uric Acid (mg/dL)	Creatinine (mg/dL)	Blood Urea Nitrogen (mg/dL)
Normal Control	2.52 ± 1.03^{a}	0.37 ± 0.03^{a}	$21.17 \pm .749^{a}$
Diabetic Control	0.10 ± 0.01^{a}	0.23 ± 0.03^{a}	52.67 ± 8.29^{b}
Diabetic Treated	0.95 ± 0.68^{a}	0.23 ± 0.09^{a}	56.5 ± 8.63^{b}

Figures that share different letter superscripts are significantly different ($P < 0.05$)

3.11. Complete Blood Count

Table 8 shows the effect of aqueous *K. pinnata* administration on corpuscular volume, erythrocyte size and hemoglobin concentration in STZ-induced diabetic rats. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and red blood cell distribution width (RDW) levels were not significantly altered by the treatment compared to the diabetic control group (Table 8).

Table 8.
Erythrocyte Indices in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata*

	MCV (fl)	MCH (pg)	MCHC (%)	RDW (%)
Normal Control	54.8 ± 0.00*	19.7 ± 0.00*	35.9 ± 0.00*	13.0 ± 0.00*
Diabetic Control	53.3 ± 1.11	18.6 ± 0.47	34.9 ± 0.15	16.2 ± 0.46
Diabetic Treated	55.0 ± 0.25	19.6 ± 0.25	35.6 ± 0.75	14.1 ± 3.00

***Due to procedural error we were only able to utilize one control sample for CBC analysis**

MCV= Mean Corpuscular Volume, MCH= Mean Corpuscular Hemoglobin, MCHC= Mean Cell Hemoglobin Concentration, RDW= Random Distribution of Width

Values were not significantly different among the groups ($P > 0.05$)

Table 9 shows the relative concentrations of red blood cells (RBC), white blood cells (WBC), platelet cells (PLT), hemoglobin (HGB) and hematocrit (HCT) levels in STZ-induced diabetic rats administered *K. pinnata* aqueous preparation. The platelet level was reduced in the diabetic control group compared to the diabetic treated group. Previous studies have shown that diminished platelet survival may account for the reduced platelet level in diabetic patients [118,119]. Platelets are essential in blood clotting and the observed increase in platelet level in the diabetic treated group may be indicative of improved platelet survival and fewer chances of uncontrolled bleeding. Red blood cell, white blood cell, hemoglobin, and hematocrit levels were not altered among the two groups (diabetic control and diabetic treated groups).

Table 9.
Blood Cell Counts in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata*

	WBC (thou./cu. mm)	RBC (mil/cu. mm)	HGB (g/dL)	HCT (%)	PLT (thou./cu. mm)
Normal Control	9.17 ± 0.00*	8.61 ± 0.00*	16.90 ± 0.00*	47.20 ± 0.00*	610.00 ± 0.00*
Diabetic Control	6.05 ± 1.24	8.70 ± 0.08	16.15 ± 0.35	46.30 ± 0.88	395.00 ± 25.20
Diabetic Treated	6.10 ± 2.55	8.60 ± 0.22	16.80 ± 0.15	47.30 ± 1.40	652.70 ± 167.00

***Due to procedural error we were only able to utilize one control sample for CBC analysis**

WBC= White Blood Cell, RBC= Red Blood Cell, HGB= Hemoglobin, HCT= Hemocrit, PLT= Platelet

Values were not significantly different among the groups ($P > 0.05$)

3.12. Inflammatory Response

Interleukins are cytokines that activate and modulate both the innate and adaptive immune system. Interleukin 1β, IL-6 and TNF-α are pro-inflammatory cytokines [120]. The trio has been found to be elevated during aging and diabetes [121].In this study, we investigated the effect of *K. pinnata* aqueous preparation on IL-1β, TNF-α, and IL-6 levels in STZ-induced diabetic rats. Low level of IL-6 has also been shown to be elevated during the acute phase response (APR), which could be triggered by diabetes and subsequently lead to the release of effector molecules that might cause endothelial dysfunction leading to atherosclerosis [122,123]. The acute phase response is the sum of complex systemic and metabolic changes that are activated due to infection, tissue injury, immunological disorders and inflammatory stimulus [124]. In this study, we noted a significant ($P < 0.05$) decrease in IL-6 in the diabetic treated group compared to the diabetic control group (Figure 17). However, we observed a non-significant up-regulation of IL-1β and TNF-α in the diabetic treated group compared to the diabetic control group. We hypothesize that the observed significant down-regulation of IL-6 in the diabetic treated group ameliorates the adverse effects associated with the observed non-significant up-regulation of IL-1β and TNF-α, which may account for our observation that *K. pinnata* preparation may protect against cardiovascular risk associated with diabetes mellitus.

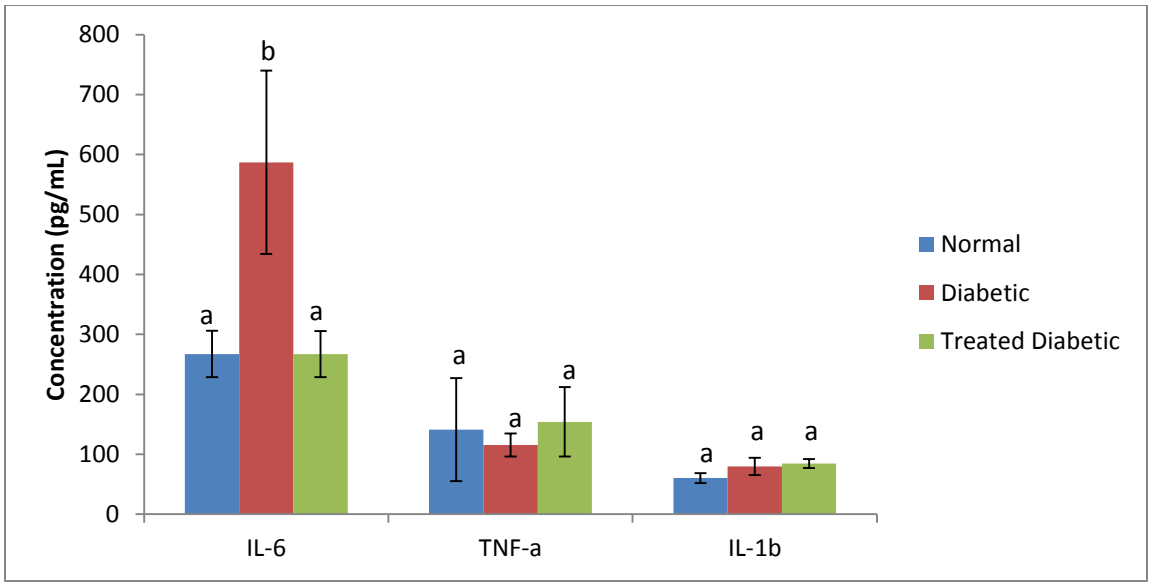

Fig. 17 Interleukin Levels in the Serum of Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata.* Figures that share different letter superscripts are significantly different ($P < 0.05$)

3.13 Table of Results

Table 10.
Summary of Changes in Some Analytes in Diabetic Rats Administered Aqueous Preparation of *Kalanchoe pinnata* Compared to the Diabetic Control Group

Fraction tested	Assay Conducted	Significance	Effect due to treatment compared to the diabetic control
Serum Glucose Level			
Serum	Glucose	Yes	Decreased
Serum Protein Profile			
Serum	Albumin	No	Increased
Serum	Globulin	No	Decreased
Serum	Album:Globulin Ratio	No	Increased
Lipid Profile			
Serum	Triglycerides	Yes	Decreased
Serum	Total Cholesterol	No	Decreased
Liver	Triglycerides	No	Decreased
Kidney	Total Cholesterol	Yes	Decreased
Kidney	HDL-Cholesterol	No	Decreased
Kidney	Triglycerides	No	Decreased
Effect on Oxidative Stress			
Serum	Catalase	Yes	Increased
Serum	Glutathione	No	Increased
Serum	Superoxide Dismutase	No	Increased
Digestive Enzymes			
Serum	Amylase	No	Increased
ATPase Activity			
Proximal Intestinal Mucosa	Na/K ATPase	No	Decreased
Proximal Intestinal Mucosa	Ca ATPase	No	Decreased
Distal Intestinal Mucosa	Mg ATPase	Yes	Increased
Distal Intestinal Mucosa	Ca ATPase	No	Decreased
Erythrocyte Membrane	Mg ATPase	Yes	Increased
Erythrocyte membrane	Na/K ATPase	No	Increased
Metabolic Enzymes			
Liver	Pyruvate Kinase	Yes	Increased
Kidney	Pyruvate Kinase	No	Decreased

Kidney	NADP-Isocitrate Dehydrogenase	Yes	Increased
Liver Function Enzymes			
Serum	Alanine Amino Transferase	No	Decreased
Serum	Aspartate Amino Transferase	No	Decreased
Kidney Function Enzymes			
Serum	Uric Acid	No	Increased
Serum	Blood Urea Nitrogen	No	Increased
Complete Blood Count			
Total Blood	Platelet level	No	Increased
Total Blood	Random Distribution of Width	No	Decreased
Inflammatory Response			
Serum	Interleukin-6	Yes	Reduced
Serum	Tumor Necrosis Factor-α	No	Increased
Serum	Interleukin-1β	No	Increased

4. CONCLUSIONS

In this study, we investigated the acclaimed traditional use of *K. pinnata* preparation in the treatment of diabetes mellitus. The plant used for this study is commonly found in tropical regions around the world. It is easy to grow and simple to prepare for therapeutic use. With the cost of pharmaceuticals on the rise, identification of a potent herbal remedy is vital in the future development of an inexpensive and effective anti-diabetic treatment. We noted three main benefits of diabetic treatment with aqueous *K. pinnata* preparation: improved hypoglycemic response, reduced cardiovascular risk factors, and an improved antioxidant defense system.

Our findings suggest that the consumption of aqueous *K. pinnata* preparation may increase hepatic glucose utilization for energy generation with subsequent decrease in blood glucose level. The increase in glycolytic activity acts to reverse the metabolic abnormalities associated with diabetes mellitus. Insulin resistance, especially in type 2 diabetes, is associated with a marked increased risk of cardiovascular disease. In this study, we noted that triglyceride and total cholesterol levels were reduced in the serum, kidney and liver of the diabetic group treated with *K. pinnata* preparation. Improved level of albumin by the treatment may be suggestive of reduced level of glycated albumin, and subsequent reduction in the risk for the development of macrovascular disease. We also noted that IL-6, which was found to be significantly elevated in the diabetic control group, was reduced to normal level due to the treatment, which may be indicative of reduced inflammation and reduced chances of vascular disease.

Hyperglycemia leads to increased production of reactive oxygen species that play a major role in the development of secondary complications in diabetes mellitus and cardiovascular disease. Our data also showed that the consumption of *K. pinnata* preparation improves antioxidant enzyme activities in the serum. Additionally, increased activity of $NADP^+$-isocitrate dehydrogenase in the kidney may represent an alternate pathway for NADPH production. NADPH is an essential cofactor for glutathione reductase and thioredoxin reductase, which play critical roles in protection against oxidative injury. While the main pathway for NADPH production (pentose phosphate pathway) remained depressed following the treatment, the observed increase in cytosolic $NADP^+$-isocitrate dehydrogenase activity may represent a compensatory pathway for oxidative reduction. We also noted an increase in serum ALP and BUN levels, perhaps indicative of tissue damage in the diabetic treated group compared to the other groups. However, these parameters can be highly nonspecific in terms of organ function.

Kalanchoe pinnata leaves have been reported to contain flavonoids, polyphenols, triterpenoids of β-amyrin structure phytosterols etc. [125,126]. These chemical constituents are speculated to account for the anti-nociceptive, anti-inflammatory and anti-diabetic activities of the herb's leaf aqueous extract [72].

Recommendations for future studies:

1) Study the effects of long-term consumption of aqueous *K. pinnata* preparation on liver and kidney function indices.
2) Study the effect of aqueous *K. pinnata* preparation consumption on hepatic phospholipid distribution with a view to establishing the probable role of the supplement on hepatic uptake of nutrients.
3) Elucidate the long-term effect of *K. pinnata* administration on key hepatic enzymes of the pentose phosphate pathway in diabetes mellitus.
4) Study the effect of aqueous *K. pinnata* preparation on pancreatic morphology and insulin receptor sites in normal and type 2 diabetic rats.
5) Identify the specific compounds in the aqueous *K. pinnata* preparation that may be responsible for the hypoglycemic, hypocholesterolemic and antioxidant activities identified in this study.

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