https://dx.doi.org/10.4314/ijs.v19i1.11
Ife Journal of Science vol. 19, no. 1 (2017)

EFFECT OF PARTIALLY PURIFIED ANGIOTENSIN CONVERTING ENZYME INHIBITORY PROTEINS FROM *MORINGA OLIEFERA* LEAVES ON ALLOXAN-INDUCED DIABETIC RATS

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(Received: 31st August, 2016; Accepted: 14th June, 2017)

ABSTRACT

This study evaluated the effect of partially-purified angiotensin converting enzyme (ACE) inhibitory proteins obtained from the leaves of *Moringa oleifera* on blood glucose, serum ACE activity and lipid profile of alloxan-induced diabetic rats. Twenty-five apparently healthy male albino rats were divided into five groups of five rats each. Groups II, III, IV and V were given 100 mg/kg alloxan intraperitoneally to induce diabetes. Rats in group I served as control, group II (diabetic control), groups III, IV and V were orally administered with glucophage (36.43mg/kg), enalapril (3mg/kg) and partially-purified proteins from *Moringa oleifera* leaves (5mg/kg), respectively, for fourteen days. The blood glucose concentration of rats given alloxan was determined 48 hours after administration of alloxan to confirm diabetes (glucose concentration $\geqslant$180mg/dl), after which treatment began. The blood glucose concentration of rats in all groups was determined on the 7th and 14th day of treatment. On the fourteenth day, the rats were sacrificed and blood collected for lipid profile and ACE analysis. Results obtained shows blood glucose level of rats given glucophage, enalapril and the partially-purified proteins from *Moringa oleifera* leaves (5mg/kg) was significantly ($p<0.05$) lower than rats in the diabetic control group. There was no significant difference ($p>0.05$) when serum glucose of treated rats was compared to the control group. The serum ACE activity of diabetic control rats (group II) increased significantly ($p<0.05$) compared to control rats. However, treatment given to rats in groups III, IV and V decreased ACE activity significantly ($p<0.05$). The serum levels of total cholesterol (TC), triglyceride and low density lipoprotein (LDL) cholesterol increased significantly ($p<0.05$), while high density lipoprotein (HDL) cholesterol decreased significantly ($p<0.05$) in diabetic rats compared to rats in control group and those given glucophage and enalapril. It is concluded from the results of this study that the partially-purified proteins from *Moringa oleifera* leaves possess hypoglycemic, hypolipidemic and ACE inhibitory activity *in vivo*.

Keywords: *Moringa oleifera*, partially-purified ACE inhibitory proteins, diabetes, angiotensin converting enzyme, lipid profile

INTRODUCTION

Diabetes mellitus and hypertension are among the commonest non-communicable diseases in both developed and developing countries (Mufunda *et al*., 2006). Diabetes is an independent risk factor for cardiovascular disease, with the risk markedly increased in the presence of hypertension. Furthermore, the overlap between diabetes and hypertension in the same individuals confers a greater risk of target organ damage, general disability and premature mortality. Hypertension affects up to 70% of individuals with diabetes and approximately twice as common in individuals with diabetes as in those without (Katte *et al*., 2014).

Diabetes mellitus (DM) is a chronic disorder characterized by impaired metabolism of glucose and lipids due to defect in insulin secretion (beta cell dysfunction) or action (insulin resistance) or both. It is the fourth leading cause of death globally (Kowluru and Chan, 2007), and one of the most challenging problems of the 21st century. According to the WHO (2016), there was an estimated 422 million adults globally living with diabetes in 2014, compared to 108 million in 1980. Also, the global prevalence (age-standardized) of diabetes has nearly doubled since 1980, rising from 4.7% to 8.5% in the adult population. The disease has a complex etiology with interacting genetic and lifestyle factors including adiposity, physical activity and diet (Brito *et al*., 2009). Diet is one of the key lifestyle factors involved in the genesis, prevention, and control of diabetes (Azadbakht *et al*., 2011).

Angiotensin I-converting enzyme (ACE) inhibitors have been reported to reduce mortality

in patients with hypertension (Vark *et al*., 2012). Compared to chemosynthetic drugs, ACE inhibitors derived from natural sources such as food proteins are believed to be safer for consumption with less adverse effects (Lau *et al.,* 2012). These drugs act as vasodilators by reducing the levels of angiotensin II in the renin angiotensin system or by inhibiting the degradation of bradykinin in the kallikrein-kinin system (Erdös, 2006). They have been prescribed as first-line treatment for hypertension in patients with type 1 diabetes, proteinuria or left ventricular systolic dysfunction (LVSD) (Flint, 2004).

It is well recognized that diabetes mellitus is associated with an increase in kidney size, in both humans and experimental animals (Glastras *et al.,* 2016). The morphologic changes that occur in diabetic organs have been best characterized in the kidney. Early in the course of diabetes, kidney cells undergo hypertrophy and later on, hyperplasia (Glastras *et al.,* 2016). There are indications of a possible role for the renin angiotensin system (RAS) in the pathogenesis of glomerular injury in diabetes mellitus (Thaiss *et al*., 1996). Also, RAS has important haemo-dynamic and growth-enhancing properties, which may play a role in development of diabetic micro-vascular complications, with conflicting results of studies on activity of ACE, the major enzyme regulating angiotensin II production (Bor *et al*., 2000).

Humans have utilized natural products since ancient times for the treatment of various diseases. The complexity, chemical diversity and biological properties of natural products during the last 200 years has led to the discovery of new drugs for the treatment of several diseases. In the last 30 years, the development of new biotechnology methods, guided phytochemical studies, automated high throughput screening and high performance analytical methods, have introduced new concepts and possibilities of rational drug design and drug discovery (Aline *et al*., 2013). About eighty percent of the world depends on herbal-based alternative method of medicine. Except for homeopathy, the activities of these curative plants are evaluated by their chemical components (Daniel, 2005).

Moringa oleifera belongs to *Moringaceae* family and originated from the Himalayan tract (Mendieta-Araica *et al*., 2012). It is made up of 13 species from tropical and subtropical climates and range in size from tiny herbs to big trees. The most widely cultivated species is *Moringa oleifera*, a multipurpose tree native to the foothills of the Himalayas in northwestern India and cultivated throughout the tropics (Marczak *et al*., 2003). In India and other countries, immature fruits, fresh leaves and flower of this tree are used for culinary purpose (Oduro *et al*., 2003). The seed contains about 35–45% edible, highly nutritious, odourless and colourless oil (Ayerza, 2012). The leaves and seeds of *M. oleifera* have been demonstrated to possess several medicinal properties. Despite the great economic importance, this plant is still unexploited fully (Pandey *et al.,* 2011). A study by Abdulazeez *et al.* (2015) reported the ACE inhibitory activity of proteins from the leaves and seeds of the plant, but very little has been documented on the effect of these proteins in diabetic animals. Therefore, this study was aimed at determining the effect of ACE inhibitory proteins obtained from the leaves of *M. oleifera* on blood glucose levels, lipid profile and ACE activity in alloxan-induced diabetic rats.

MATERIALS AND METHODS

Materials

Chemicals and reagents

Alloxan Monohydrate, Enalapril, Glucophage, HEPES sodium salt, Sodium Chloride, Hippuryl-L-Histidyl-L-Leucine and Angiotensin Converting Enzyme were obtained from Sigma Chemical Company (St. Louis, MO, USA). Other reagents including Disodium hydrogen tetraoxophosphate (VI), Potassium dihydroxyl tetraoxosulphate (VI) acid and Ethyl Acetate were of analytical grade.

METHODS

Experimental Animals

Twenty-five (25) apparently healthy male albino rats weighing between 130 -190 g were purchased from the animal house in the Zoology Department, Faculty of Science, Bayero University, Kano, Nigeria. The animals were kept in well-ventilated cages in the animal house and allowed access to both food and water *ad libitum* throughout the period of study. All experimental protocols were approved and conducted with

strict adherence to guidelines and procedures of the Institutional Animal Care and Use Committee of Bayero University, Kano.

Induction of Diabetes

All animals, except those in group I (control group) were made diabetic by a single intraperitoneal injection of alloxan monohydrate (100 mg/kg). The rats were confirmed to be diabetic when the glucose concentration was ⩾ 180mg/dl. They were allowed 72 hours of rest for blood glucose stabilization before the administration of the drugs. The initial blood glucose of each rat was measured before induction and also on the 7th and 14th days of treatment.

The rats were divided into five (5) groups of five (5) rats each:

Group I: Served as the control, not diabetic and given distilled water.

Group II: Positive control, diabetic and untreated

Group III: Diabetic and given 36.43 mgkg^{-1} body weight of standard drug (glucophage)

Group IV: Diabetic and given 3 mg kg^{-1} body weight of enalapril

Group V: Diabetic and given 5mg kg^{-1} body weight of partially purified proteins from *Moringa oleifera* leaves.

Partial-Purification of ACE Inhibitory Proteins from *Moringa oleifera*

This was done as described by Abdulazeez *et al.* (2015). Fresh *Moringa oleifera* leaves were washed with distilled water, pounded and macerated in phosphate buffer (pH 7.4). The mixture was then centrifuged at 4 °C and 10000xg for 15 minutes to obtain the supernatant, which was precipitated using cold acetone at a ratio of 1:4. Then the test-tube containing the precipitated protein was vortexed and incubated at -20 °C for 60 minutes after which it was centrifuged for 10 minutes at 4 °C and 10000xg and supernatant was discarded leaving the pellet with acetone allowed to evaporate from the uncapped test-tube. The pellet was reconstituted in 5 ml phosphate buffer (pH 7.4) to determine the ACE inhibitory activity and protein content. The proteins were consequently partially-purified using gel filtration and ion exchange chromatography. The fractions with the highest ACE inhibitory activity were pulled together, concentrated under reduced pressure using a rotary evaporator, lyophilized and stored at 4 °C until needed.

Fasting blood glucose determination

The glucose oxidase method was used to determine blood glucose using a glucometer (Accu-check one-touch basic) and results were expressed in mg/dl.

Collection and Preparation of Samples

The animals were treated for 14 days. On the 14th day, they were sacrificed by anesthesia and blood collected in well-labeled test tubes. The serum was obtained and used for all analysis carried out.

Lipid Profile Determination

Serum total cholesterol, triacylglycerol and high-density lipoprotein cholesterol were determined using Randox Laboratory kit reagents, while Low-density lipoprotein (LDL) cholesterol was determined by differential subtraction of the sum of the cholesterol fractions from the total cholesterol. The absorbances of all the tests were determined using spectrophotometer (HAICH, DR 3000, Germany).

Determination of ACE Activity

This was determined as described by Cushman and Cheung (1971). About 50 µl of serum was added to 50 µl deionized water and the reaction started by adding 0.2 ml of 5 mmol/L hippuric-histidyl-leucine (HHL). This was incubated at 37 °C for 15 minutes. The reaction was terminated by adding 0.25 ml of 1.0 N hydrochloric acid and then 2.0 ml ethyl acetate to extract the hippuric acid formed by the action of ACE. This was centrifuged at 3600 x g for 2 min, and 1 ml of upper layer transferred into a microcentrifuge tube and heated by dry bath at 100 °C for 15 minutes to remove ethyl acetate by evaporation. The resulting hippuric acid was dissolved in 3.0 ml of distilled water, and the absorbance read at 228 nm. Serum enzyme activity was expressed in units, which corresponded to 1 µmol of hippuric acid released by hydrolysis of HHL per minute per milliliter serum.

Statistical analysis

All data were presented as mean ± SD of each group. They were analysed by one-way ANOVA using GraphPad Instat3 Software (2000) version

3.05 by GraphPad Inc. Values of $P < 0.05$ were considered significant.

RESULTS

Forty eight (48) hours after administration of alloxan monohydrate, the blood glucose level of rats in groups II, III, IV and V increased significantly ($p<0.05$) except that for the control rats (group I) that were not induced. Thus, there was a significant difference ($p<0.05$) between the control rats and those in all other groups (Table 1).

Table 2 shows the blood glucose concentration of rats in all groups after 7 and 14 days treatment. From the results, treatment of diabetic rats significantly ($p<0.05$) reduced blood glucose after 7 and 14 days compared to the levels of blood glucose after induction. Despite the reduction in blood glucose one week after commencement of treatment, the blood glucose level of control rats was significantly ($p<0.05$) lower than that of rats in all treated groups. Rats in diabetic control group had significantly ($p<0.05$) higher blood glucose level than other groups, both after the 7th and 14th days of treatment. The blood glucose level of rats given Glucophage (145.50±3.42 and 89.25±1.71 mg/dl) was significantly ($p<0.05$) lower than those given enalapril (179.25 ± 6.70 and 101.75 ± 6.29 mg/dl) and *M. oleifera* ACE inhibitory proteins (185.75±6.55 and 111.50 ± 5.07 mg/dl) after seven and fourteen days of treatment, respectively. However, there was no significant ($p>0.05$) difference in glucose levels of rats given enalapril and *M. oleifera* ACE inhibitory proteins after two weeks of treatment (Table 2).

Table 1: Blood Glucose Level of Rats Before and After Induction of Diabetes

Groups	Blood glucose level before induction (mg/dl)	Blood glucose level 48 Hrs after induction (mg/dl)
Control	92.50 ± 18.93[a]	95.00 ± 9.13[a]
Diabetic Control	91.00 ± 6.38[a]	353.00 ± 2.94[b]
Glucophage (36.43 mg/kg)	98.75 ± 15.48[a]	307.00 ± 4.76[c]
Enalapril (3 mg/kg)	86.25 ± 4.50[f]	338.75 ± 34.73[d]
M. *oleifera* proteins (5 mg/kg)	94.75 ± 17.42[g]	338.25 ± 94.19[c]

Values are mean ± standard deviation. Values with different superscript on the same row are significantly different at $p<0.05$.

Table 2: Effect of Partially-Purified ACE Inhibitory Proteins from M. *oleifera* Leaves on Blood Glucose Levels of Alloxan-Induced Diabetic Rats

Groups	Day 7 (mg/dl)	Day 14 (mg/dl)
Control	95.25 ± 4.11[a]	95.75 ± 13.96[a]
Diabetic Control	351.25 ± 41.57[c]	378.75 ± 23.23[b]
Glucophage (36.43mg/kg)	145.50±3.42[c]	89.25 ± 1.71[d]
Enalapril (3 mg/kg)	179.25 ± 6.70[d]	101.75 ± 6.29[e]
M. *oleifera* proteins (5mg/kg)	185.75±6.55[d]	111.50 ± 5.07[c]

Values are mean ± standard deviation. Values with different superscript down the column are significantly different at $p<0.05$.

Results of the effects of partially-purified proteins from leaves of *M. oleifera* on lipoprotein levels of alloxan-induced diabetic rats are presented on table 3. The results showed that induction of diabetes significantly ($p<005$) increased blood levels of total cholesterol (2.99 ± 0.13 mmol/L), triacylglycerol (1.72 ± 0.40 mmol/L) and LDL cholesterol (1.01 ± 0.05 mmol/L), and decreased HDL cholesterol (1.38 ± 0.11mmol/L) compared to the control rats and those given glucophage and enalapril. There was no significant ($p>0.05$) difference in LDL cholesterol levels when control rats were compared to those given glucophage and enalapril, while total cholesterol decreased significantly ($p<0.05$) in the treated rats compared to control. On the other hand, HDL levels were

higher ($p<0.05$) in rats given glucophage (1.68 ± 0.06 mmol/L) than the control group (1.59 ± 0.30mmol/L). Although the partially-purified proteins from the leaves of *M. oleifera* significantly ($p<0.05$) reduced total cholesterol and LDL cholesterol compared to the diabetic control rats, the level of total cholesterol in these rats was significantly ($p<0.05$) higher than rats given glucophage and enalapril, but not different ($p>0.05$) from control rats. There was no significant ($p>0.05$) difference in LDL cholesterol level of rats given the partially-purified proteins, those given glucophage and enalapril and those in control group. The HDL and triacylglycerol levels of rats given the partially purified proteins from leaves of *M. oleifera* were not significantly ($p>0.05$) different from diabetic control rats.

Table 4 shows the serum angiotensin converting enzyme activity of diabetic rats treated with glucophage, enalapril and partially-purified proteins from leaves of *M. oleifera*. There was no significant ($p>0.05$) difference in serum ACE activity of diabetic and control rats. However, treatment of rats with all three drugs significantly ($p<0.05$) reduced ACE activity compared to control and diabetic control rats. There was no significant ($p>0.05$) difference between rats treated with Glucophage, enalapril and proteins from leaves of *M. oleifera* proteins.

Table 3: Effect of Partially Purified ACE Inhibitory Proteins from Leaves of M. *oleifera* on Lipoprotein Levels of Alloxan-Induced Diabetic Rats

Groups	Total Cholesterol (mmol/L)	HDL (mmol/L)	Triacylglycerol (mmol/L)	LDL (mmol/L)
Control	2.40 ± 0.44^{e}	1.59 ± 0.30^{k}	0.64 ± 0.28^{a}	0.70 ± 0.33^{a}
Positive control	2.99 ± 0.13^{w}	1.38 ± 0.11^{d}	1.72 ± 0.40^{p}	1.01 ± 0.05^{b}
Glucophage (36.43 mg/kg)	2.13 ± 0.06^{a}	1.68 ± 0.06^{b}	1.17 ± 0.11^{n}	0.64 ± 0.16^{a}
Enalapril (3 mg/kg)	2.28 ± 0.08^{a}	1.56 ± 0.47^{k}	1.49 ± 0.31^{b}	0.51 ± 0.04^{a}
M. *oleifera* (5 mg/kg)	2.51 ± 0.19^{e}	1.46 ± 0.06^{d}	1.77 ± 0.30^{p}	0.60 ± 0.06^{a}

Values are mean ± standard deviation. Values with different superscript down the column are significantly different at $p<0.05$

Table 4: Effect of Partially Purified ACE Inhibitory Proteins from Leaves of M. *oleifera* on Serum Angiotensin Converting Enzyme Activity of Alloxan-Induced Diabetic Rats

Groups	ACE Activity (µmol/ML)
Control	0.703 ± 0.327^{a}
Diabetic control	1.010 ± 0.005^{a}
Glucophage (36.43 mg/kg)	0.643 ± 0.057^{b}
Enalapril (3 mg/kg)	0.513 ± 0.035^{b}
M. oleifera proteins (5mg/kg)	0.600 ± 0.060^{b}

Values are mean ± standard deviation. Values with different superscript down the column are significantly different at $p<0.05$.

DISCUSSION

The most important biological marker used in the diagnosis and monitoring of diabetes mellitus both in clinical and experimental settings is the measurement of blood glucose (Mayfield, 1998). In the present study, the significant increase in serum glucose level in diabetic rats compared to control rats shows that administration of alloxan monohydrate was effective in inducing hyperglycemia, which has been attributed to the damage of the pancreatic beta cells by alloxan (Szudelski, 2001). Alloxan monohydrate is one of the drugs used to induce diabetes mellitus, by damaging insulin-secreting cells of the pancreas leading to hyperglycemia (Szudelski, 2001). Treatment of diabetic rats with the standard diabetes drug, glucophage, also known as metformin, significantly reduced blood glucose levels of rats. Metformin decreases hepatic glucose production and intestinal absorption of glucose, and improve insulin sensitivity by increasing peripheral glucose uptake and utilization (Graham *et al*., 2013).

The significant reduction in blood glucose levels after treatment with enalapril and partially purified proteins from leaves of *M. oleifera* conforms to several studies (de Kloet *et al*., 2009; Mastan *et al*., 2011) showing that ACE inhibitors reduce blood glucose level. Angiotensin-converting enzyme (ACE) inhibitors have been used for treating refractory hypertension since the early eighties, and since then, clinical investigations support the benefits of ACE inhibitors in pathologies like congestive heart failure, myocardial infarction, diabetes mellitus, chronic renal insufficiency, and atherosclerotic cardiovascular disease (Maria *et al.,* 2009). A study by McLaughlin *et al.* (2009) demonstrated that calcium antagonists (another antihypertensive drug class) are less effective than ACE inhibitors in preventing cardiovascular events in diabetic hypertensive patients. The 'appropriate blood pressure control in diabetes' trial group also found that diabetic patients treated with ACE inhibitors had lower incidence of myocardial infarction and overall cardiac events (Estacio and Schrier, 1998). The blood glucose lowering effect of these inhibitors have been attributed to their protective action on skeletal muscle and pancreatic islets, enhanced insulin sensitivity and increased transcapillary glucose transport (Ribeiro-Oliveira *et al*., 2008).

The high total cholesterol, LDL and triacylglycerol levels in diabetic rats supports the results of Mendez and Balderas (2001) and Mironova *et al.* (2000), and has been attributed to increase in the mobilization of free fatty acids from the peripheral fat depots, since insulin inhibits the hormone-sensitive lipase (Shanmugasundaram *et al*., 2011). The increased fatty acid concentration also increases the alpha-oxidation of fatty acids, producing more acetyl Co-A and cholesterol during diabetes. Under normal conditions, insulin increases receptor-mediator removal of LDL-cholesterol, and decreased activity of insulin during diabetes causes hypercholesterolemia. Hypercholesterolemia and hypertriglycedemia have been reported to occur in diabetic rats (Shanmugasundaram *et al*., 2011), as is also evident in this study (Table 3). The reduction of total cholesterol, LDL and triacylglycerol, and increased HDL cholesterol compared to diabetic rats by enalapril agrees with studies showing that ACE inhibitors improve lipid profile by increasing HDL-cholesterol and decreasing triacylglycerols and cholesterol levels. They maintain normal endothelial function, with preserved vasodilatation response (Ferrari *et al*., 2010) and increase circulating endothelial progenitor cells (EPC) by improving lipid status, blockade of production of aldosterone and the conversion of angiotensin I to angiotensin II (Godfrey *et al*., 1994). This could be a plausible explanation for the reduction in total cholesterol and LDL cholesterol in rats given the partially-purified proteins from leaves of *M. oleifera*, although the mechanism is not well understood. This benefit of ACE inhibitors has not been universal, as there are reports that they do not have effect on lipid profile (Williams *et al*., 2006; Krysiak and Okopień, 2008).

Diabetes mellitus is associated with diffuse vascular damage in vascular beds which has been suggested may either contribute to or be caused by the alterations of RAS in the circulation. The increase in ACE activity on induction of diabetes may be through the diffuse vascular damage that occurs in diabetes, causing the release of ACE (Edmund *et al*., 1993), although the exact mechanism remains to be classified.

CONCLUSION

In conclusion, this study has demonstrated that the partially-purified ACE inhibitory proteins from leaves of *Moringa oleifera* have *in vivo* ACE inhibitory activity. It also possesses significant hypoglycemic and hypolipidemic effects, and thus its potential as a promising candidate for management of diabetic and hypertensive patients.

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