Original Article

Moringa olifeira Lam. Stimulates Activation of the Insulin-Dependent Akt Pathway. Antidiabetic Effect in a Diet-Induced Obesity (DIO) Mouse Model

(*Moringa olifeira* / insulin resistance / hyperglycaemia / hyperinsulinaemia / DIO mice / Akt / GLut4 / SREBP-1 / PPARα)

E. S. ATTAKPA[1, 3*], M. M. SANGARÉ[1], G. J. BÉHANZIN[1], J.-M. ATEGBO[1], B. SERI[2], N. A. KHAN[3]

[1]Laboratoire de Physiopathologie Moléculaire et Toxicologie, Département de Physiologie Animale, Faculté des Sciences et Techniques, Université d'Abomey Calavi Cotonou, Rép. du Bénin
[2]Laboratoire de Neurosciences, Unité de Formation Biosciences 22 BP 582 Abidjan 22, Université de Cocody-Abidjan, Rép. de Côte-d'Ivoire
[3]INSERM U866, Physiologie de la Nutrition & Toxicologie. Université de Bourgogne, Dijon, France

Abstract. We investigated the antidiabetic effect of *Moringa olifeira* Lam. in a diet-induced obesity (DIO) mouse model. Six mice were randomly selected as normal controls. *Moringa olifeira* Lam. leaf extract at a dose of 200, 400 or 600 mg/kg body weight, glibenclamide (Glib) at the dose of 10 mg/kg (positive control) and distilled water at 10 ml/kg (control group) were administered orally by gastric intubation, and each group consisted of six mice. Insulin-sensitive tissues (liver, skeletal muscle) were collected to investigate antidiabetic effects and examine the plant's molecular mechanisms. *Moringa olifeira* Lam. leaf extract prevented weight gain. It also reduced blood glucose in DIO mice. Glib and *Moringa olifeira* Lam. leaf extract, 400 mg/kg, treatments restored insulin levels towards normal values ($P < 0.05$ versus diabetic control group). Western immunoblot analysis of different tissues, collected at the end of the study, demonstrated that *Moringa olifeira* Lam. stimulated activation of the insulin-dependent Akt pathway and increased the protein content of Glut 4 in skeletal muscle. The improvement of hepatic steatosis observed in DIO-treated mice was associated with a decrease in the hepatic content of SREBP-1, a transcription factor involved in *de novo* lipogenesis. The hepatic PPARα protein content in the plant extract-treated mice remained significantly higher than those of the control group ($P < 0.05$). In conclusion, this study provides the first evidence for direct action of *Moringa olifeira* Lam. on pancreatic β-cells, enhancing glucose-stimulated insulin secretion. This correlated with hypoglycaemic effects in diabetic mice associated with restored levels of plasma insulin.

Introduction

Obesity is indeed a major risk factor for insulin resistance and type 2 diabetes. Worldwide, the number of people with diabetes and pre-diabetes is exponentially increasing mainly due to aging, urbanization, unhealthy eating habits, increasing prevalence of obesity and lack of physical activity (Wild et al., 2004). Diabetes mellitus is a leading cause of morbidity and mortality worldwide.

Because of the high cost of conventional treatments with synthetic drugs, traditional treatment with plants becomes an alternative option for financially poor populations who may have problems of accessibility to modern drugs. For these reasons, the development of new therapies from plants that are able to control diabetes mellitus is of great interest.

The medicinal properties of this plant depend on the part of the plant concerned (root, leaf stalk and pulp or fruit) and the extract used (ethanolic, butanolic, aqueous extract, etc.). *Moringa olifeira* Lam. (Moringaceae) is a highly valued plant, distributed in many countries of the

Received June 29, 2016. Accepted January 21, 2017.

Corresponding author: Eugène Sèlidji Attakpa, Laboratoire de Physiopathologie Moléculaire et Toxicologie, Département de Physiologie Animale, Faculté des Sciences et Techniques, Université d'Abomey Calavi Cotonou, Rép. du Bénin. Phone: (+229) 97-55-21-25; e-mail: eattakpa@yahoo.fr

Abbreviations: ACC – acetyl-CoA carboxylase, Akt – protein kinase B, DIO – diet-induced obesity, FAT – fatty acid transport, FAS – fatty acid synthase, Glib – glibenclamide, Glut4 – glucose transporters, HFD – high-fat diet, IKK – inhibitor of κB kinase, MO – *Moringa olifeira* Lam., PPARα – peroxisome-proliferator-activated receptor α, SREBPs – sterol regulatory element-binding proteins, Wat – white adipose issue.

tropics and subtropics. It has an impressive range of medicinal uses with high nutritional value. Different parts of this plant contain a profile of important minerals, and are a good source of protein, vitamins, β-carotene, amino acids and various phenolics. The Moringa plant provides a rich and rare combination of zeatin, quercetin, β-sitosterol, caffeoylquinic acid and kaempferol. In addition to its compelling water-purifying powers and high nutritional value, *Moringa olifeira* Lam. is very important for its medicinal value. Various parts of this plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods act as cardiac and circulatory stimulants, possess anti-tumour, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol-lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities, and are being employed for the treatment of different ailments in the indigenous system of medicine, particularly in South Asia. This review focuses on the detailed phytochemical composition, medicinal uses, along with pharmacological properties of different parts of this multipurpose tree.

Insulin resistance is characterized by a decrease in the ability of target tissues, especially muscle, liver and adipose tissue, to respond to insulin effects. Therefore, at normal concentrations, insulin is unable to produce an adequate biological response. To compensate, insulin-secreting β pancreatic cells increase their release of the hormone, thus resulting in a state of hyperinsulinaemia. Eventually, β-cell dysfunction leads to impaired insulin secretion, which accentuates deregulation of glucose and lipid metabolism in insulin-sensitive tissues such as skeletal muscle, adipose tissue and liver. The muscle is the main site of glucose disposal in human, and approximately 80 % of total body glucose uptake occurs in skeletal muscle (Pan et al., 1997) through insulin- and exercise-sensitive glucose transporters, Glut4. Following exercise or insulin stimulation, Glut4 transporters translocate from intracellular vesicles (basal state) to the cell surface of muscle cells (and, to a lesser extent, of adipose cells) to mediate glucose uptake from the bloodstream, without necessarily altering expression levels of the transport protein. The insulin-dependent Akt pathway (Brassard et al., 1993) can modulate Glut4 translocation. In addition, manipulation of Glut4 levels in transgenic mice revealed that glucose homeostasis is also highly sensitive to Glut4 expression levels. In fact, specific elevation in the level of Glut4 expression in the muscle prevents insulin resistance (Spoor et al., 2006).

In the present study, we tested these key components of muscle glucose homeostasis, Akt, and Glut4, in skeletal tissues of obese and insulin-resistant mice treated with *Moringa olifeira* Lam., a promising antidiabetic. Adipose tissue is a major site of fatty acid synthesis and storage. White adipose tissue (WAT) also plays an important role in regulating systemic insulin sensitivity by secreting adipokines such as adiponectin and leptin, which influence whole body metabolism (Jiang et al., 2003).

In the present study, we therefore paid attention to peroxisome-proliferator-activated receptor α (PPARα), C/EBP, adiponectin and leptin parameters. The liver plays a crucial role in the homeostasis of glucose by its ability to control the blood sugar level through glucose production or storage, notably in the form of glycogen. This organ also regulates lipid homeostasis through a process implicating key lipogenic enzymes such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), as well as transcription factor sterol response element-binding protein-1 (SREBP-1) (Wallberg-Henriksson et al., 2001). The latter controls synthesis of cholesterol, fatty acids and triglycerides (Aledo et al., 1997) and may be involved in the pathogenesis of hepatic insulin resistance (Karlsson et al., 2005) through enhancement of lipogenenic enzymes. This leads to intracellular accumulation of triglycerides and hence hepatosteatosis (Wallberg-Henriksson et al., 2001). The increase of SREBP-1 expression is usually caused by hyperinsulinaemia and inflammatory cytokines such as inhibitor of κB kinase (IKK) (Ploug et al., 1984; Nesher et al., 1985). SREBP1c controls transcription of lipogenic genes. Hence, SREBP-1 and PPARα have been assessed in this study to determine the effects of *Moringa oleifera* on the liver *in vivo*. PPARα is principally expressed in organs with a high capacity for fatty acid oxidation, e.g., heart, skeletal muscle, liver, and kidney (Wahli et al., 2002; Zambon et al., 2006). The chemical constituents of *Moringa oleifera* Lam. consist mainly of flavonoids and phenolic compounds that participate in the plant's medicinal proprieties (Baumann et al., 2000). We therefore sought to confirm the plant's antidiabetic potential *in vivo* and to further investigate the mechanisms by which this plant can improve systemic glucose and lipid homeostasis. For this purpose, we chose the diet-induced obesity (DIO) mouse model. Indeed, mice fed chronically with a high-fat diet develop obesity, hyperglycaemia and hyperlipidaemia (Cano et al., 2007). The diet-induced-obesity (DIO) model thus adequately reflects the fact that a high-fat diet is the major environmental factor causing overweight and participating in the metabolic syndrome to type 2 diabetes continuum in humans (Kersten et al., 2001).

In order to induce insulin resistance, most of the investigators have adopted a strategy in which they feed the rodents with the high-fat (HF) diets (Pagliassoti et al., 1994; Buettner et al., 2007).

Material and Methods

Plant material

Moringa olifeira Lam. was collected from the southeastern part of Benin (Abomey-Calavi, in Department of Atlantic) between mid-July and mid-August 2015 during the short dry season when the mean temperature equals to 28 ± 2 °C. This period is preceded by the great rain season (mid-March to mid-July). The plant was recognized by the Principal Botanist of the National Herba-

rium of Benin of the University of Abomey-Calavi, which contained the voucher specimen (AP-2078-HNB).

Preparation of plant material extract

Powdered leaves (1.2 kg) were extracted by percolation using 70% (v/v) ethanol, and the mixture was then filtered using Whatmann filter paper No. 1. The extract was dried by evaporation using rotary vaporizers under reduced pressure at a temperature of 40–45 °C. The residue filtrate obtained was then dried by steam bath at 40 °C and kept in a refrigerator at 8 °C for experimental usage. The yield of the aqueous ethanol extract was 20.1 % by weight (w/w).

Solvent fractionation of the total ethanol extract

The procedure for solvent-solvent separation was adopted from Ranjan et al. (2002) with minor modifications. Ten percent (w/v) of ethanol extract of the plant was prepared with mildly hot distilled water. The dissolved aqueous ethanol extract was separated in a separatory funnel with 50 ml of n-hexane (3 times), and extracted in 50 ml of dichloromethane (3 times) and 50 ml of n-butanol (3 times) successively, until the extracting solvent became colourless. After completing the separation process, the solvents were removed by a rotary evaporator. The separated n-butanol fraction was dried by steam bath at 40 °C and kept in the refrigerator for the experiments. The percentage yield of n-butanol was 7.8 (w/w).

Animals

Four-week-old male C57BL/6 mice were purchased from Charles River (Les Oncins, France), housed in separate cages and kept in a temperature and humidity controlled room with a 12h light-dark cycle. They were fed with commercial chow diet for two days to stabilize their metabolic condition and allow them to adjust to the new environment.

DIO mouse model

Four-week-old male C57BL/6 mice (Charles River) were housed in separate cages and kept in a temperature and humidity controlled room with a 12h light-dark cycle. They were fed with commercial chow diet for two days to stabilize their metabolic condition and allow them to adjust to the new environment. Then, the mice were randomly divided into five groups (N = 6/group):

– One group received standard chow diet (Charles River) throughout the 16-week study (CHOW control group).
– One group received a high-fat diet (HFD) throughout the 16-week study (DIO-control group).
– The other three groups received HFD for 8 weeks until they became obese and insulin resistant. The treatment protocol was then initiated by adding the *Moringa olifeira* Lam. leaf extract into the HFD at three different doses (200, 400, 600 mg/kg) for another 8 weeks. Diets were prepared freshly every week and stored at 4 °C.

The control diet was a standard laboratory chow (Teklad 8604, Teklad, Madison, WI), containing 4.7 % fat by weight and 14 % kcal from fat, 32 % kcal from protein, and 54 % kcal from carbohydrate. The high-fat diet was Teklad TD.07011, containing 29 % fat by weight and 54 % kcal from fat (comprised of hydrogenated vegetable shortening and corn oil), 21 % kcal from protein, and 24 % kcal from carbohydrate. Diets were prepared freshly every week and stored at 4 °C. The food and water were provided *ad libitum* and replenished every 3–4 days. During the study, body weight, food intake, water intake and blood glucose levels were measured two or three times a week, at the same time in the morning.

The blood glucose levels were determined by the glucose oxidase method. At the end of the treatment study, the mice were anaesthetized (intraperitoneal sodium pentobarbital, 45 mg/kg), sacrificed by exsanguination, and organs such as the liver, skeletal muscle, were immediately collected, weighed and stored at –80 °C for further analysis. The experiments complied with the local institutional ethical guidelines.

Effect of Moringa olifeira Lam. leaf extract treatment assessed by the glucose tolerance test in DIO mice

Moringa olifeira Lam. leaf extract at a dose of 200, 400 or 600 mg/kg body weight, glibenclamide (Glib) at the dose of 10 mg/kg (positive control) and distilled water at 10 ml/kg (control group) were administered orally by gastric intubation, and each group consisted of six mice. Doses of the *Moringa olifeira* Lam. leaf extract were selected based on previous animal studies (Ojewole et al., 2003; Dimo et al., 2007) and used by traditional practitioners. Chronic treatment was conducted by daily administration of the test compounds for 14 consecutive days with measurements of body weight and fasting glycaemia at the indicated times. Acute effects were evaluated in fasted animals during a glucose tolerance test. Immediately after oral glucose (5 g/kg) administration, the test compounds were administered as described above, and glycaemia was recorded over a 5-h period.

Biochemical assays

On the last day of the study, animals in each group were anesthetized with pentobarbital (60 mg/kg body weight). The abdominal cavity was opened, and whole blood was drawn from the abdominal aorta. Serum was obtained by low-speed centrifugation (1000 $g \times 20$ min) and immediately used for glucose testing. Some aliquots were stored at –80° C for insulin determination.

The determination of insulin was performed in samples that were stored at -80 °C. Serum insulin was determined using an ELISA kit (LINCO Research Inc, St. Charles, MO), according to the manufacturer's instructions. Serum glucose was determined by the glucose oxidase method using a glucose analyser (Beckman Instruments, Brea, CA). Adiponectin and leptin (Millipo-

re, St. Charles, MO) were determined using radioimmunoassay kits according to manufacturer's specifications.

Western blot analysis

Frozen tissue (muscle, liver) samples were homogenized in RIPA lysis buffer (50 mM Hepes, 150 mM NaCl, 5 mM EGTA, 2 mM MgCl2, 5% glycerol, 1% Triton-X 100, 0.1% SDS, pH 7.4) containing protease and phosphatase inhibitors (2 mM PMSF, 10 Mm NaF, 100 µM Na-orthovanadate, 1 mM Na-pyrophosphate). After homogenization, samples were centrifuged at 12,000 *g* for 12 min at 4 °C and supernatant was then removed and stored at –80 °C until analysis. The total protein content of each sample was quantified using the Bradford method. Equal amounts of protein (50 µg) were subjected to electrophoresis in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Millipore, Bedford, MA). Membranes were first blocked for 2 h at room temperature in 5% non-fat dry milk in TBST (20 mM Tris; pH 7.6, 137 mM NaCl, 0.1% Tween-20), then incubated with primary antibodies overnight at 4 °C. The membranes were washed three times with TBST and then incubated with the secondary antibody for 1 h at room temperature. The signal was revealed by the ECL Plus Western blotting detection system (Perkin Elmer, Woodbridge, Canada). Membranes were probed with the following antibodies; p-Akt (Ser 473), protein kinase B (Akt), ß-actin (1 : 1000 dilution, 5% BSA, Cell Signaling Technology, Danvers, MA); GLut4, PPARα, SREBP-1 (1 : 200 dilution; 5% milk, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The anti-rabbit IgG HRP-conjugated secondary antibodies or anti-mouse IgG HRP-conjugated secondary antibodies were used at 1 : 4000 dilutions in 5% milk in TBST (Cell Signaling Technology).

Results

Diet-induced-obesity (DIO)

The DIO group was fed with the high-fat diet for a period of 16 weeks to induce obesity and a pre-diabetic state. In the DIO group animals, we observed that this resulted in a significant weight gain as compared to the standard diet-fed group (50.8 ± 0.4 g vs 38.5 ± 0.2 g, respectively; $P < 0.05$). Serum glucose concentration was significantly higher in the DIO mice in comparison with their corresponding controls.

In parallel, a state of insulin resistance was established as evidenced by mild hyperglycaemia and major hyperinsulinaemia in DIO mice (9-fold increases, 5.20 ng/ml ± 0.75 vs 46.80 ng/ml ± 17.26, respectively; $P < 0.05$). Finally, the circulating leptin/adiponectin ratio, also indicative of insulin resistance, significantly increased from 1.21 ± 0.1 in the controls to 2.52 ± 0.21 in DIO animals ($P < 0.05$).

Effect of Moringa olifeira Lam. leaf extract treatment assessed by the glucose tolerance test in DIO mice

Fasted untreated mice with stabilized diabetes received one single acute treatment immediately after oral glucose load, and glycaemia was monitored over 5 h (Fig. 1). Animals received the glucose load and the treatment simultaneously, both by gavage. Glib rapidly lowered plasma glucose close to non-diabetic levels (171 ± 9 mg/dl at 60 min), an effect maintained over the 5-h recording period. *Moringa olifeira* Lam. leaf extract at 200 mg/kg also reduced glycaemia towards non-diabetic levels, although the effects were slightly delayed compared with Glib. At 400 mg/kg, the *Moringa olifeira* Lam. leaf extract treatment significantly reduced plasma

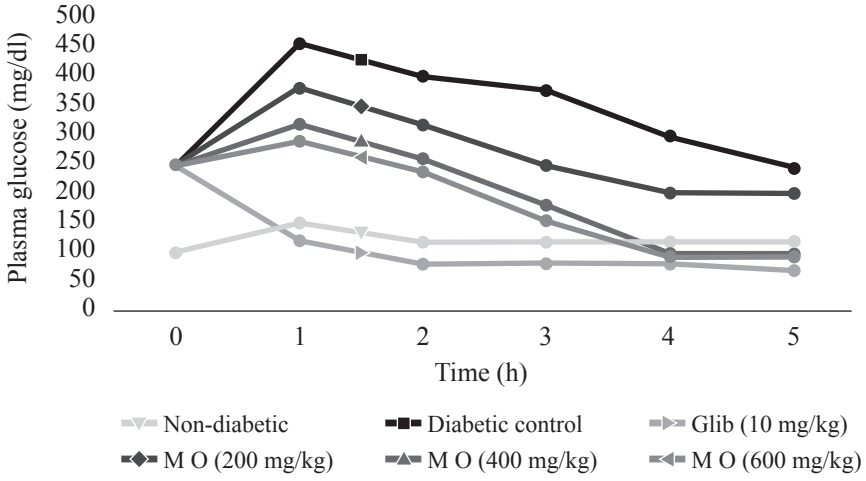

Fig. 1. Effect of *Moringa olifeira* Lam. leaf extract treatment on glucose tolerance test in DIO mice
DIO group animals were fed with high-fat diet for a period of 16 weeks to induce obesity and a pre-diabetic state. Fasted untreated mice with stabilized diabetes received one single acute treatment immediately after oral glucose load and glycaemia was monitored over 5 h. $P < 0.05$ versus diabetic control, N = 6

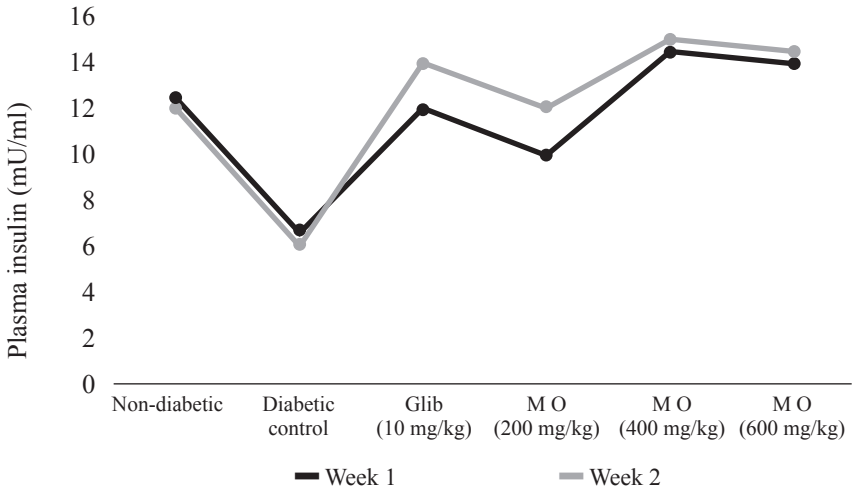

Fig. 2. Effect *of Moringa olifeira* Lam. leaf extract treatment on insulin levels in DIO mice
DIO group animals were fed with high-fat diet for a period of 16 weeks to induce obesity and a pre-diabetic state. After 14 days of indicated daily treatments, fasted rats were killed, and plasma insulin levels were determined. $P < 0.05$ versus diabetic control, $N = 6$

glucose levels at all time points, resulting in glycaemia of 180 ± 10 mg/dl at 5 h (–45 % versus diabetic controls, $P < 0.05$). Moringa *olifeira* Lam. leaf extract effects were not statistically different between 400 mg/kg and 600 mg/kg.

Effect of Moringa olifeira Lam leaf extract treatment on insulin levels in DIO mice

At the end of the 2-week treatment period, plasma insulin concentrations in diabetic controls were reduced to 6.0 ± 1.3 compared with 12.0 ± 0.9 mU/ml in non-diabetic animals ($P < 0.05$), see Fig. 2. Both Glib and *Moringa olifeira* Lam. leaf extract, 400 mg/kg, treatments restored insulin levels towards normal values ($P < 0.05$ versus diabetic control group). These data show efficient hypoglycaemic effects of chronic *Moringa olifeira* Lam. leaf extract treatment correlating with increased circulating insulin.

Effect of Moringa olifeira Lam. leaf extract treatment on glycaemia in diabetic mice

Fasting plasma glucose levels were dramatically increased two weeks after diabetes induction by DIO, reaching values of 252 ± 13 mg/dl in diabetic controls (Fig. 3). After 7 days of daily treatment, both Glib and *Moringa olifeira* Lam. leaf extract, 400 mg/kg, markedly reduced glycaemia. *Moringa olifeira* Lam. leaf extract at the dose of 200 mg/kg was also efficient in correcting

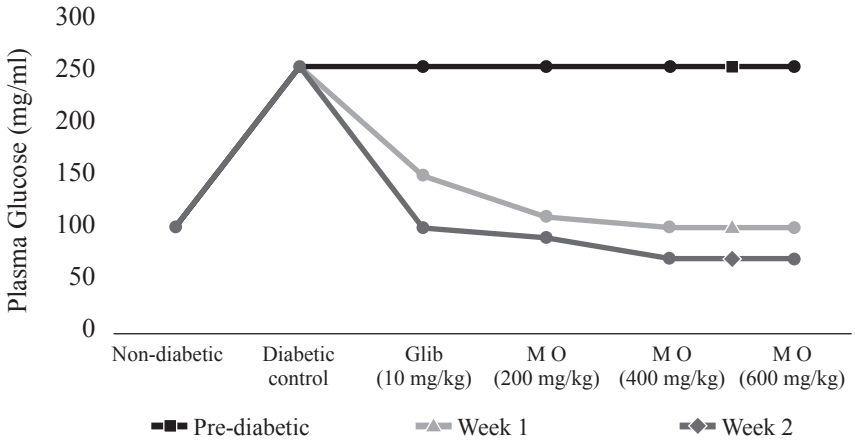

Fig. 3. Treatment of diabetic mice with *Moringa olifeira* Lam. leaf extract
Effects of chronic *Moringa olifeira* Lam. leaf extract treatment on glycaemia in diabetic rats: the diet-induced-obesity (DIO) mouse model, which is based on feeding the high-fat diet at pre-diabetic time; initiation of treatments (day 1). Daily oral administration of water (non-diabetic and diabetic control), of *Moringa olifeira* Lam. leaf extract (200, 400 and 600 mg/kg) and of glibenclamide (10 mg/kg, Glib) was continued for 14 days. Fasting glycaemia was recorded. $P < 0.05$ versus diabetic control, $N = 6$

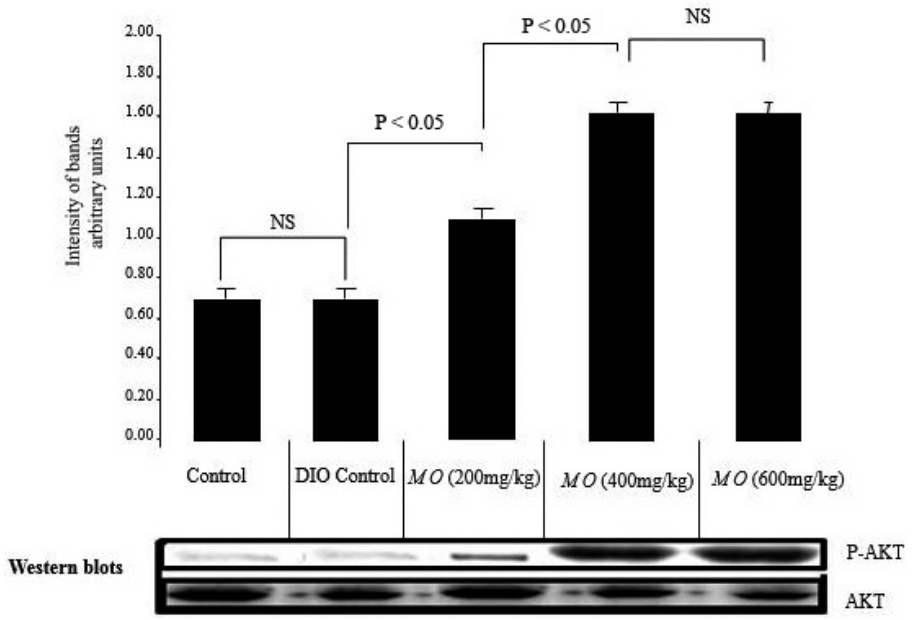

Fig. 4. Moringa olifeira stimulates the Akt pathway in the muscle.
The diet-induced-obesity (DIO) mouse model, which is based on feeding the high-fat diet (at pre-diabetic time). Samples of skeletal muscle (50 µg protein) from mice fed control chow, DIO and DIO + *Moringa olifeira* Lam. leaf extract (200, 400 and 600 mg/kg) were homogenized and analysed by immunoblotting as described in the Material and Methods section. Representative immunoblots and their quantification are shown for samples probed with p-Akt (Ser 473)/Akt.
Values are means ± SEM from 6 animals in each group. P value ≤ 0.05 indicates significant difference. NS – non-significant differences

plasma glucose when compared with diabetic controls (150 ± 9 vs 250 ± 10 mg/dl, $P < 0.05$). After 15 days of treatment, both Glib and *Moringa olifeira* Lam. leaf extract groups exhibited glycaemia similar to non-diabetic mice compared with diabetic controls ($P < 0.05$).

Moringa olifeira Lam. stimulates the Akt pathway in the muscle

The insulin-dependent Akt pathway known to be involved in hepatic glucose and lipid homeostasis was also assessed. As illustrated in Fig. 4, *Moringa olifeira* Lam. treatment was found to activate the insulin-dependent Akt pathway. Densitometric analysis of Western blots revealed that, when compared to control DIO mice, *Moringa oleifera* Lam. extract dose-dependently increased the protein level of Akt.

Moringa olifeira Lam. upregulates glucose transporter Glut4 expression through an Akt-dependent pathway in the muscle

To begin elucidating the mechanisms of action of *Moringa olifeira* Lam. responsible for the observed systemic metabolic effects in the DIO mouse model, we analysed the signalling pathways involved in glucose uptake in the muscle. Figure 5 shows that *Moringa olifeira* Lam. increased the muscle Glut4 protein levels in mice ($P < 0.05$).

Moringa olifeira Lam. stimulates PPARα and SREBP-1 in the liver

The liver PPARα content was significantly increased in DIO-control animals as compared to the controls ($P < 0.05$; Fig. 6), suggesting compensatory mechanisms in the face of enhanced fatty acid intake. *Moringa olifeira* Lam. treatment upregulated hepatic PPARα levels beyond those of the DIO-control animals. Nevertheless, the hepatic PPARα protein content in the plant extract-treated mice remained significantly higher than those of the control group ($P < 0.05$). In the case of SREBP-1, the levels of the 68 kDa active fragments increased in the DIO-control animals ($P < 0.05$; Fig. 7). Interestingly, *Moringa olifeira* Lam. treatment decreased p68 SREBP-1 levels as compared to the DIO-control group ($P < 0.05$).

Discussion

In the present study, the antidiabetic activity was investigated in the DIO mouse model. Treatment with *Moringa olifeira* Lam. was administered for 8 weeks, after obesity and mild hyperglycaemia were established by an initial 8 weeks of HFD feeding. Diabetes mellitus is a metabolic disorder that usually affects carbohydrate, fat, and protein metabolism, followed by multi-organ dysfunction in the later period, and hyperlipidaemia associated with hyperglycaemia (Upendra et al., 2010). Effective novel compounds with pan-target antidiabetic

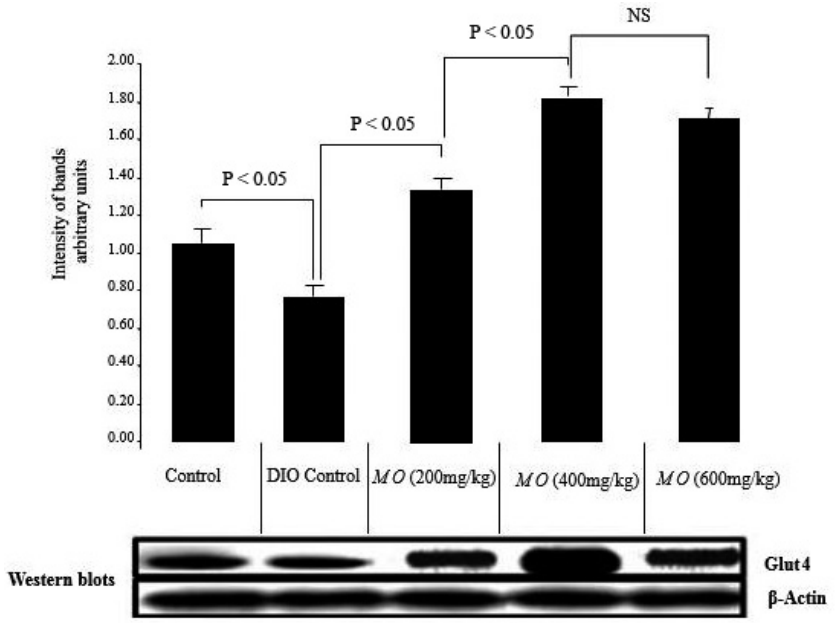

Fig. 5. Moringa olifeira Lam. upregulates glucose transporter Glut4 expression through an Akt-dependent pathway in the muscle.

The diet-induced-obesity (DIO) mouse model, which is based on feeding the high fat diet (at pre-diabetic time). Samples of skeletal muscle (50 µg protein) from mice fed control chow, DIO and DIO + *Moringa olifeira* Lam. leaf extract (200, 400 and 600 mg/kg) were homogenized and analysed by immunoblotting as described in the Material and Methods section. Values are means ± SEM from 6 animals in each group. Representative immunoblots and their quantification are shown for samples probed with GLUT4/β-actin. P value ≤ 0.05 indicates significant difference. NS – non-significant differences

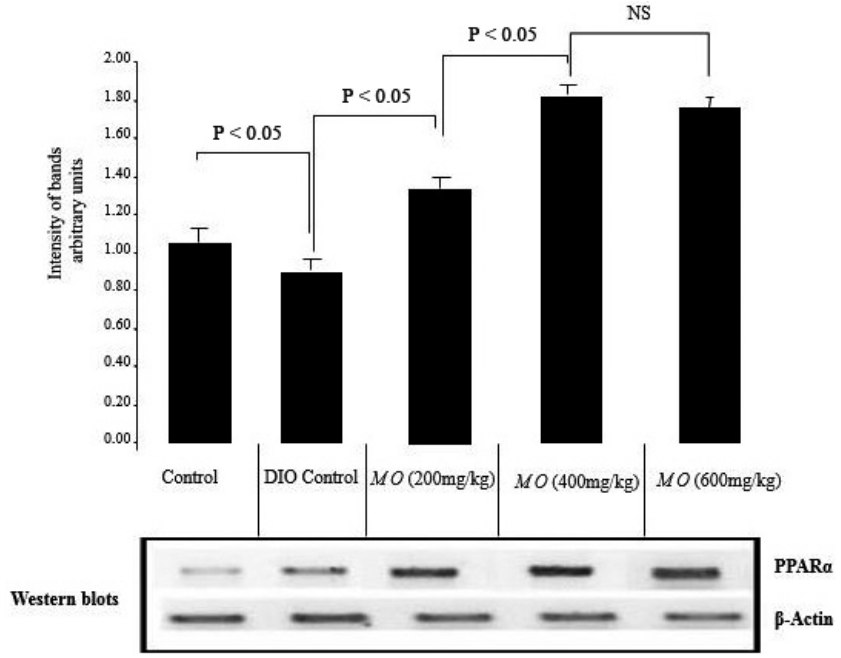

Fig. 6. Moringa olifeira Lam. stimulates PPARα in the liver.

The diet-induced-obesity (DIO) mouse model, which is based on feeding the high-fat diet (at pre-diabetic time). Samples of liver tissue (50 µg protein) from mice fed control chow, DIO and DIO + *Moringa olifeira* Lam. leaf extract (200, 400 and 600 mg/kg) were homogenized and analysed by immunoblotting as described in the Material and Methods section. Values are means ± SEM from 6 animals in each group. Representative immunoblots and their quantification are shown for samples probed with PPARα/β-actin. P value ≤ 0.05 indicates significant difference. NS – non-significant differences

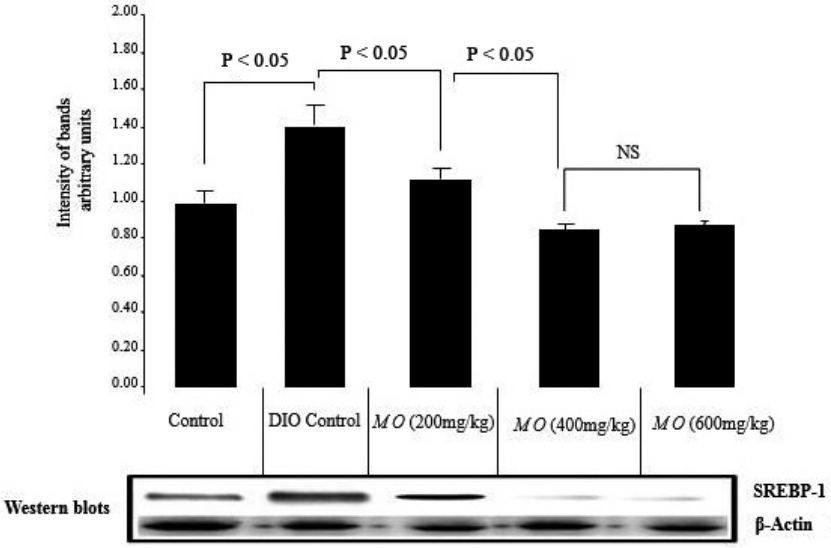

Fig. 7. Moringa olifeira Lam. stimulates SREBP-1 in the liver of DIO mice.
The diet-induced-obesity (DIO) mouse model, which is based on feeding the high-fat diet (at pre-diabetic time). Samples of liver tissue (50 µg protein) from mice fed control chow, DIO and DIO + *Moringa olifeira* Lam. leaf extract (200, 400 and 600 mg/kg) were homogenized and analysed by immunoblotting as described in the Material and Methods section. Values are means ± SEM from 6 animals in each group. Representative immunoblots and their quantification are shown for samples probed with SREBP-1. P value ≤ 0.05 indicates significant difference. NS – non-significant differences

activity and proven long-term safety should be targeted in a clinical setting for patients with coexisting relevant lipid and glucose metabolic disorders. These discoveries pave the way for the development of drugs for treating chronic multigenic metabolic and cardiovascular diseases, for which therapy is presently insufficient or non-existent (Mukherjee et al., 2006; Toma et al., 2013). This is the first study to investigate the effect of aqueous ethanol and n-butanol fractions of *Moringa oleifera* Lam. leaves on the mechanisms aimed at attenuation of insulin resistance through an Akt-dependent pathway in the muscle in the DIO mouse model. The presence of phytochemicals in plant products offers a great potential for balancing metabolic disturbances.

Several phytomolecules, including flavonoids, total phenolic compounds, alkaloids, glycosides, saponins, glycolipids, dietary fibres, polysaccharides, peptidoglycans, carbohydrates, amino acids and others obtained from various plant sources, have been reported as potent hypoglycaemic and anti-hyperglycaemic agents. Flavonoids are a heterogeneous group of ubiquitous plant polyphenols, which exhibit a variety of pharmacological activities, including the anti-atherogenic as well as anti-hyperglycaemic effects, lipoprotein oxidation, blood platelet aggregation and vascular reactivity (Kumar et al., 2010; Brahmachari et al., 2011). A high content of phytochemicals, especially total polyphenolic compounds and total flavonoids, may contribute to the pleiotropic effects of *Moringa olifeira* Lam. leaves that support the use of the plant for different metabolic disorders in the local community (Toma et al., 2014). A high content of total phenolic compounds and flavonoids may have a significant role in regulating metabolic disturbances that are highly related to diabetes mellitus and its complications due to its protective effect on the pancreas and other essential organs.

The results clearly demonstrate that such treatment improves glucose homeostasis in the face of continued HFD feeding and strongly suggest that this is achieved by an attenuation of insulin resistance. Indeed, *Moringa olifeira* Lam. treatment significantly countered hyperglycaemia.

Plasma insulin levels were increased by the *Moringa olifeira* Lam. leaf extract treatment *in vivo*, indicating an action on pancreatic β-cells. Accordingly, we then investigated the putative direct effects of *Moringa olifeira* Lam. leaf extract on β-cells.

Noteworthy, the *Moringa olifeira* Lam. leaf extract efficiently reduced glycaemia at 200 mg/kg, resulting in glycaemia of 180 ± 10 mg/dl at 5 h (–45 % versus diabetic controls, $P < 0.05$), although plasma insulin levels were similar to diabetic controls. This suggests that tissues other than pancreatic β-cells also contributed to the lowering of the blood glucose.

The *Moringa olifeira* Lam. leaf extract effects were not statistically different between 400 mg/kg and 600 mg/kg doses. Indeed, the *Moringa olifeira* Lam. leaf extract treatment was shown previously to increase hepatic glycogen storage to a similar extent as metformin (Gondwe et al., 2008). Such liver-specific effects could possibly explain the blood glucose lowering effect observed in mice as soon as 1 h after the first administration of *Moringa olifeira* Lam. leaf extract. Among other molecules, *Moringa olifeira* Lam. leaf extract contains epicatechin-3-galloyl ester (Galvez Peralta et al., 1992) that is also present in green tea and has been shown to

improve glucose tolerance in diabetic mice and human subjects (Tsuneki et al., 2004).

The major organs involved in the regulation of blood glucose levels are the muscle, the liver and the adipose tissue. Therefore, activation and expression of the key proteins involved in glucose and lipid homeostasis were assessed in these insulin-sensitive tissues in order to begin elucidating the molecular mechanisms underlying the apparent systemic antidiabetic activity of *Moringa olifeira* Lam.

Firstly, in the muscle, the expression of Glut4 glucose transporters increased up to two-fold with *Moringa olifeira* Lam. treatment as compared to DIO-control mice. This is consistent with the literature, where specific elevation of Glut4 expression in the muscle was described to prevent insulin resistance and enhance its sensitivity in normal mice (Spoor et al., 2006).

In addition, this increase in muscle Glut4 levels was associated with activation of the insulin-dependent Akt pathway. It is well documented that ectopic fat accumulation in the liver is a major contributor to the development of insulin resistance in this organ and that this is a crucial component in diabetes pathogenesis (Samuel et al., 2004). Conversely, liver insulin sensitivity is enhanced by stimulating hepatic fatty acid oxidation through AMPK/ACC (Winder et al., 1999; Foretz et al., 2006) and/or PPARα (Poynter et al., 1998; Fatehi-Hassanabad et al., 2005) activities, on the one hand, and by inhibiting cholesterol and triglyceride synthesis through regulation of SREBP-1, on the other (Yahagi et al., 2002; Kohjima et al., 2008). Results obtained in the DIO-control mice were associated with increased SREBP-1. On the other hand, the enhanced PPARα levels observed in DIO-control animals suggest that compensatory mechanisms may have been induced in the face of sustained high intake in lipids. The results obtained in *Moringa olifeira* Lam.-treated mice indicate that the plant extract can also improve hepatic lipid homeostasis. As mentioned, the *Moringa olifeira* Lam. treatment significantly elevated levels of PPARα were maintained and SREBP-1 levels reduced in *Moringa olifeira* Lam.-treated mice as compared to DIO-control congeners; these data suggest that *Moringa olifeira* Lam. treatment could tip the balance towards increased oxidation of fatty acids and less lipid storage in the liver, thus improving hepatic steatosis and hence insulin sensitivity. These results therefore support our interpretation that *Moringa olifeira* Lam. probably exerts its antidiabetic activity by acting mainly on the liver and muscle.

Acknowledgements

The authors thank the Ministry of Higher Education and Research of Benin. We also express our sincere thanks to the French Ministry of Research, which granted a contingent grant for the laboratory (Laboratoire de Physiopathologie Moléculaire et Toxicologie). We highly appreciate the contribution of Professor Naim Akhtar Khan, who established the collaboration between Dijon (France) and Cotonou (Benin).

Discloser of conflict of interest

All of the authors have nothing to declare as far as the conflict of interest is concerned.

Authors' contributions

Eugène Sèlidji Attakpa designed the study, wrote the manuscript, and was in charge of the major parts of practical work and participated in the interpretation of data. Maxime Machioud Sangaré, Gbèssohélè Justin Béhanzin and Jean-Marie Atègbo participated in the technical work and contributed to the collection of plant materials and their extraction. Bialli Seri supervised the plant collection and extraction. Naim Akhtar Khan participated in the final drafts of the manuscript.

References

Aledo, J., Lavoie, L., Volchuk, A., Keller, S. R., Klip, A., Hundal, H. S. (1997) Identification and characterization of two distinct intracellular GLUT4 pools in rat skeletal muscle: evidence for an endosomal and an insulin-sensitive GLUT4 compartment. *Biochem. J.* **325**,727-732.

Baumann, C. A., Ribon, V., Kanzaki, M., Thurmond, D. C., Mora, S., Shigematsu, S., Bickel, P. E., Pessin, J. E., Saltiel, A. R. (2000) CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature* **14**, 202-207.

Brahmachari, G. (2011) Six bio-flavonoids with promising antidiabetic potentials: a critical survey. In *Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry.* Ed. V. K. Tiwari, pp. 187-212, Research Signpost, Kerala, India.

Brassard, P., Robinson, E., Lavallee, C. (1993) Prevalence of diabetes mellitus among the James Bay Cree of northern Quebec. *CMAJ* **3**, 303-307.

Buettner, R., Schölmerich, J., Bollheimer, L. C. (2007) High-fat diets: modeling the metabolic disorders of human obesity in rodents. *Obesity (Silver Spring)* **15**, 798-808.

Cano, N. B. D., Schneider, S. M., Vasson, M. P., Hasselmann, M., Leverve, X. (2007) *Traité de nutrition artificielle de l'adulte.* Springer-Verlag, Paris, France. (in French)

Dimo, T., Rakotonirina S. V., Tan P. V., Azay, J., Dongo, E., Kamtchouing, P., Cros, G. (2007) Effect of Sclerocarya birrea (Anacardiaceae) stem bark methylene chloride/methanol extract on streptozotocin-diabetic rats. *J. Ethnopharmacol.* **110**, 434-438.

Fatehi-Hassanabad, Z., Chan, C. B. (2005) Transcriptional regulation of lipid metabolism by fatty acids: a key determinant of pancreatic β-cell function. *Nutr. Metab. (Lond.)* **5**, 21-31.

Foretz, M., Taleux, N., Guigas, B., Horman, S., Beauloye, C., Andreelli, F., Bertrand, L., Viollet, B. (2006) Regulation of energy metabolism by AMPK: a novel therapeutic approach for the treatment of metabolic and cardiovascular diseases. *Med. Sci. (Paris)* **22**, 381-388.

Galvez Peralta, J., Zarzuelo, A., Busson, R., Cobbaert, C., de Witte, P. (1992) (K)-Epicatechin-3-galloyl ester: a secretagogue compound from the bark of Sclerocarya birrea. *Planta Medica* **58,** 174-175.

Gondwe, M., Kamadyaapa, D. R., Tufts, M., Chuturgoon, A. A., Musabayane, C. T. (2008) Sclerocarya birrea [(A. Rich.) Hochst.] [Anacardiaceae] stem-bark ethanolic extract (SBE) modulates blood glucose, glomerular filtration rate (GFR) and mean arterial blood pressure (MAP) of STZ-induced diabetic rats. *Phytomedicine* **15**, 699-709.

Jiang, G. Z. B. (2003) Glucagon and regulation of glucose metabolism. *Am. J. Physiol. Endocrinol. Metab*. **4**, E671-E678.

Karlsson, H. K., Zierath, J. R., Kane, S., Krook, A., Lienhard, G. E., Wallberg-Henriksson, H. (2005) Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects. *Diabetes* **6**, 1692-1697.

Kersten, S. (2001) Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep*. **2**, 282-286.

Kohjima, M., Higuchi, N., Kato, M, Kotoh, K., Yoshimoto, T., Fujino, T., Yada, M., Yada, R., Harada, N., Enjoji, M., Takayanagi, R., Nakamuta, M. (2008) SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. *Int. J. Mol. Med.* **21**, 507-511.

Kumar, S., Malhotra, R., Kumar, D. (2010) Antidiabetic and free radicals scavenging potential of Euphorbia hirta flower extract. *Indian J. Pharm. Sci.* **72**, 533-537.

Mukherjee, P., Maiti, K., Mukherjee, K., Houghton, P. (2006) Leads from Indian medicinal plants with hypoglycemic potentials. *J. Ethnopharmacol.* **106**, 1-28.

Nesher, R., Karl I. E., Kipnis, D. M. (1985) Dissociation of effects of insulin and contraction on glucose transport in rat epitrochlearis muscle. *Am. J. Physiol*. **249**, C226-C232.

Ojewole, J. A. (2003) Hypoglycemic effect of Sclerocarya birrea [(A. Rich.) Hochst.] [Anacardiaceae] stem-bark aqueous extract in rats. *Phytomedicine* **10**, 675-681.

Pagliassoti, M. J., Knobel, S. M., Shahrokhi, K. A., Manzo, A. M., Hill, J. O. (1994) Time course of adaptation to a high-fat diet in obesity-resistant and obesity-prone rats. *Am. J. Physiol*. **267**, 659-664.

Pan, X. R., Li, G. W., Hu, Y. H., Wang, J. X., Yang, W. Y., An, Z. X., Hu, Z. X, Lin, J., Xiao, J. Z., Cao, H. B., Liu, P. A., Jiang, X. G., Jiang, Y. Y., Wang, J. P., Zheng, H., Zhang, H., Bennett, P. H., Howard, B. V. (1997) Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance. The Da Qing IGT and Diabetes Study. *Diabetes Care* **4**, 537-544.

Ploug, T., Galbo, H., Richter, E. A. (1984) Increased muscle glucose uptake during contractions: no need for insulin. *Am. J. Physiol*. **6**, E726-E731.

Poynter, M. E., Daynes, R. A. (1998) Peroxisome proliferator-activated receptor α activation modulates cellular redox status, represses nuclear factor-κB signaling, and reduces inflammatory cytokine production in aging. *J. Biol. Chem.* **273**, 32833-32841.

Ranjan, C., Reeba, K., Mullangi, R., Sharma, V. M., Jagadheshan, H., Rao, Y. N. (2002) Antidiabetic and hypolipidemic activity of Helicteres isora in animal models. *J. Ethnopharmacol*. **81**, 343–349.

Samuel, V. T., Liu, Z. X., Qu, X., Elder, B. D., Bilz, S, Befroy, D., Romanelli, A .J., Shulman, G. I. (2004) Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J. Biol. Chem.* **31**, 32345-32353.

Spoor, D. C., Martineau, L. C., Leduc, C., Benhaddou-Andaloussi, A., Meddah, B., Harris, C., Burt, A., Fraser, M. H., Coonishish, J., Joly, E., Cuerrier, A., Bennett, S. A., Johns, T., Prentki, M., Arnason, J. T., Haddad, P. S. (2006) Selected plant species from the Cree pharmacopoeia of northern Quebec possess antidiabetic potential. *Can. J. Physiol. Pharmacol*. **84**, 847-858.

Toma, A. (2013) Recent advances on novel dual-acting peroxisome proliferator-activated receptor α and γ agonists. *Int. J. Pharm. Sci. Res*. **4**, 1644-1653.

Toma, A., Makonnen, E., Mekonnen, Y., Debella, A., Adisakwattana, S. (2014) Intestinal α-glucosidase and some pancreatic enzymes inhibitory effect of hydroalcholic extract of Moringa stenopetala leaves. *BMC Complement. Altern. Med*. **14**, 1472-1480.

Tsuneki, H., Ishizuka, M., Terasawa, M., Wu, J.B., Sasaoka, T., Kimura, I. (2004) Effect of green tea on blood glucose levels and serum proteomic patterns in diabetic (db/db) mice and on glucose metabolism in healthy humans. *BMC Pharmacology* **4** 18.

Upendra, M., Sreenivasulu, M., Chengaiah, B. (2010) Microvascular and macro-vascular complication of diabetes mellitus. *IJPRIF* **2**, 1883-1892.

Wahli, W. (2002) Peroxisome proliferator-activated receptors (PPARs): from metabolic control to epidermal wound healing. *Swiss Med. Wkly.* **132,** 83-91.

Wallberg-Henriksson, H., Zierath, J. (2001) GLUT4: a key player regulating glucose homeostasis? Insights from transgenic and knockout mice. *Mol. Membr. Biol.* **3**, 205-211.

Wild, S., Roglic, G., Green, A., Sicree, R., King, H. (2004) Global prevalence of diabetes. *Diabetes Care* **27**, 1047-1053.

Winder, W. W, Hardie, D. G. (1999) AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am. J. Physiol.* **277**, E1-E10.

Yahagi, N., Shimano H., Hasty, A. H., Matsuzaka, T., Ide, T., Yoshikawa, T., Amemiya-Kudo, M., Tomita, S., Okazaki, H., Tamura, Y., Lizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., Yamada, N. (2002) Absence of sterol regulatory element-binding protein-1 (SREBP-1) ameliorates fatty livers but not obesity or insulin resistance in Lep(ob)/Lep(ob) mice. *J. Biol. Chem.* **277**, 19353-19357.

Zambon, A., Gervois P., Pauletto, P., Fruchart, J. C., Staels, B. (2006) Modulation of hepatic inflammatory risk markers of cardiovascular diseases by PPARα activators: clinical and experimental evidence. *Arterioscler. Thromb. Vasc. Biol*. **26**, 977-986.