Research Article

In vitro Toxicological Evaluation and Effect of *Bridelia ferruginea Benth (Euphorbiaceae)* on Glycemia and Insulin Secretion in Healthy Rats

Batomayena Bakoma[1*], Aboudoulatif Diallo[1], Povi Lawson-evi[2], Bénédicte Berké[3], Kwashie Eklu-Gadegbeku[2], Kodjo Aklikokou[2], Edmond Creppy[4], Messanvi Gbeassor[2], Nicholas Moore[3]

[1]Department of Pharmacy, Faculty of Health Sciences, University of Lomé, Togo.
[2]Department of Physiology, Faculty of Sciences, University of Lomé, Togo.
[3]Department of Pharmacology, University of Bordeaux, Bordeaux, France.
[4]Laboratory of Toxicology and Applied Hygiene, University of Bordeaux, France.
***Corresponding author's E-mail:** bbakoma@gmail.com

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ABSTRACT

The aim of this study was to compare the mechanism of action of *Bridelia ferruginea* root bark hydro alcoholic extract to metformin and glibenclamide on diabetes and evaluate its safety *in vitro*. Materials and Methods: A single dose of *B. ferruginea* extract (250 mg/kg), metformin (100 mg) and glibenclamide (5 mg/kg) was administered to normal blood glucose level Wistar rats. Blood glucose and insulin were measured 3 hours after administration of substances. Another daily administration of substances at the same doses for 14 consecutive days was performed, blood glucose and insulin level were determined at 0, 7, 14th day. MTT and red neutral assay were performed in Vero and N2A cells for in vitro toxicological evaluation. In a single administration, we noticed that *B. ferruginea* and metformin have no significant effect on insulin secretion nor on basal blood glucose level during the 3 hours compared to rats that received distilled water. Glibenclamide significantly ($P < 0.001$) reduced blood glucose level to the third time with an increase in insulin concentration. In repeated dose, we also note that *B. ferruginea* and metformin had no effect on insulin secretion nor on basal blood glucose during the 14 days of treatment. We note a significant increase in insulin levels and reduction of blood glucose level in animals that received glibenclamide. For MTT assay, the IC_{50} was 245 µg/mL for N2A cell and 286 µg/mL for Vero cell. With the neutral red assay, IC_{50} was 211 µg/mL for N2A cells and 317 µg/mL for Vero cells. These results show that *Bridelia ferruginea* root bark hydro alcoholic extract would have a similar mechanism of action to that of metformin.

Keywords: *Bridelia ferruginea*, cytotoxicity, blood glucose and insulin.

INTRODUCTION

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels.[1]

The pharmacological treatment of diabetes includes oral hypoglycemic drugs and insulin. Although these drugs are effective in reducing glycemia, they may cause undesirable side effects (such as weight gain, hypoglycemia, edema, gastrointestinal disturbances and insulin resistance) that can discourage patient compliance. On the other hand, ethnopharmacological evidence has shown that the use of plants is a viable alternative for the treatment of diabetes. The advantages of herbal medicine include significant efficacy, low incidence of side effects, low cost and relative safety.[2] In fact, the medicinal plants are considered an important source of molecules with potential hypoglycemic effects. Some authors have reported about 800 plants with these molecules which may act through different mechanisms, including the inhibition or stimulation of enzymatic activity and/or protein expression.[3] The wide diversity of species has led scientists to make great efforts to bioprospect plants that may contribute to the management of diabetes.

The root bark of *B. ferruginea* is used for the treatment of diabetes in Togolese traditional medicine. In our previous study, the effects of *Bridelia ferruginea* hydro alcoholic extract were proven on some parameters of metabolic syndrome[4], there was lack of apparent, acute or sub-chronic toxicity at doses greater than those that induce an effect in animal disease models.[5] The ethyl acetate soluble fraction of the hydro alcoholic extract from the roots of *Bridelia ferruginea* were found to be the most active fraction.[6] Phytochemical investigations suggest that catechins can be some of active compounds.[7] The present study was designed to identified the mechanism by which *B. ferrruginea* can act on diabetes (like metformin or glibenclamide) and evaluated its safety in vitro using both the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetra-zolium bromide (MTT) and neutral red assays.

The neutral red assay was included as it does not rely on a reduction reaction to determine viability, thereby reducing the possibility of obtaining false positive results in the presence of antioxidants or other reductive agents.[8]

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MATERIALS AND METHODS

Plant Material

The roots of *Bridelia ferruginea* (Euphorbiaceae) were collected in July 2012 from Tsévié area, 35 km North East of Lomé (Togo). Botanical authentication was confirmed at the Department of Botany, University of Lomé, where a voucher specimen of *B. ferruginea* was deposited at the herbarium (TG 03068).

Preparation of Crude extract

The root barks of *B. ferruginea* were sliced, shade dried and coarsely powdered. The powder was macerated at room temperature with ethanol-water (8:2, v/v) three times during 36 hours.

The filtrate was concentrated to dryness under vacuum in a rotary evaporator at 45°C and yielded a residue of 17.5% (w/w) as compared to the initial powder weight.

Animals

Wistar rats (BW 80-85 g) purchased from Elevage Janvier (France) were maintained under standard conditions with a 12h light/dark cycle and had free access to standard laboratory diet and water. Prior to initiation of dosing, all rats were acclimated for 7 days and evaluated for weight gain and any gross signs of disease or injury.

After acclimatization, rats were randomized to different groups on the basis of their body weights using an electronic scale (Tefal, Ecully, France). All rats were housed six per group in polypropylene cages. Principles of laboratory animal care as described in the European Community guidelines were followed (Official Journal of European Union L197 vol. 50, July 2007).

Experimental Procedure

Effect of *B. ferruginea* single dose on normal rats glycemia

Animals were randomly assigned into four groups of 6 each. Group I: rats received distilled water (10 mL/kg per day), group II: rats received *B. ferruginea* root bark extract (250 mg/kg), group III: metformin 100 mg/kg, group IV: glibenclamide (5 mg/kg). Blood was collected from rats with capillary tube from retino-orbital plexus at 0, 60, 120 and 180 minutes after substances administrations.

The samples were centrifuged at 3000 g for 15 min, serum obtained was aliquoted and frozen for glucose level assay (commercial kits, BioMerieux Ref: 61 162, France) and insulin level assay (Rat and mice Insulin Enzyme Immunoassay Kit, SPI-BIO, Montigny Le Bretonneux, France).

Effect of *B. ferruginea* repeated dose on normal rats glycemia

Animals were also randomly assigned into four groups of 6 each. Group I: rats received distilled water (10 mL/kg per day). Group II: rats received *B. ferruginea* (250 mg/kg/day). Group III, metformin 100 mg/kg/day. Group IV, glibenclamide (5 mg/kg/day) for 14 day consecutively.

Blood was collected from rats with capillary tube from retino-orbital plexus at day 0, 7 and 14. Blood glucose and insulin level were determined.

In vitro toxicological assay

Cell Culture

The Vero cells (kidney epithelial cells extracted from an African green monkey) and N2A cells (mouse neuroblastoma cells) were obtained from Dr. Jing Yu, (Tufts School of Medicine; Medford, MA, USA). Cells were routinely cultured in a humidified 5% CO2–95% air mixture at 37°C and were grown in DMEM or RPMI medium (Sigma, France), supplemented with 10% foetal bovine serum, 8 mML-glutamine, penicillin (100 UI/mL) and streptomycin (100 mg/mL).

Neutral red uptake assay

Neutral red is a vital dye taken up by cells and incorporated in lysosomes. Dead or damaged cells do not take up the dye. Briefly, Cells were seeded in 96-wells microplates (10,000 cells/ 200 mL/well) and routinely cultured in a humidified incubator for 24 h. Cells were maintained in culture and exposed to plant extracts over a range of concentrations 50–500 mg/mL for N2A cells and 50-1000 mg/mL for Vero cells. After 24 h exposure to extracts, neutral red uptake test (NR) was performed according to some authors[9]. Briefly, at the end of the treatment (24 h), the medium with or without extracts was discarded, and 200 mL of freshly prepared neutral red solution (50 mg/mL) was added to each well. Cells were then re-incubated for an additional 4 h at 37°C. Thereafter, the cells were carefully washed twice with 200 mL of PBS to eliminate extracellular NR. The incorporated dye was eluted from the cells by adding 200 mL elution medium (50% ethanol supplemented with 1% acetic acid, v/v) to each well followed by gentle shaking of the microplate for 15 min. The plates were then read at 540 nm using a microplate reader (Dynatech MR 4000, Dynatech, Boston, MA, USA). Survived cells in treated wells were expressed as percentage of control wells. The IC_{50} (50% viability inhibitory effect) was determined and expressed in µg/mL.

MTT assay

The MTT assay is a test of metabolic competence based upon assessment of mitochondrial performance. It is a colorimetric assay relying on the conversion of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. This test was carried out according to the method described by Kouadio[10]. Cells were seeded in 96-wells microplates (10,000 cells/mL/well) and routinely cultured in a humidified incubator for 24 h.

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Cell culture media were removed and extracts were added in concentration ranging from 50 to 500 mg/mL for N2A cells and 50 to 1000 mg/mL for Vero cells. Cells were then incubated for 24 h. In this test, a control group (DMEM or RPMI without extract) and a blank group (without cells or medium) were also included. The medium with or without extract was then discarded, and 100 mL of tetrazolium salt MTT solution (0.5 mg/mL in DMEM or RPMI) was added to each well. Cells were re-incubated for an additional 2 h; 100 mL of 10% SDS in 0.01 M HCl was added to each well to dissolve the formazan crystals. The plates were then read on a microplate reader (DYNATECH MR 4000, Dynatech, Boston, MA, USA) at 560 nm. Four wells were used for each concentration. The IC_{50} (50% viability inhibitory effect) was determined and expressed in µg/mL.

Statistical Analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test to evaluate significant differences between groups. P < 0.05 was considered statistically significant. All statistical analyses were carried out using the Instat statistical package (Graph Pad Software Inc., USA).

RESULTS

Effect of single dose administration of substances on blood glucose and insulin level on healthy rats during 180 min.

A single administration noticed that hydro alcoholic extract of *Bridelia ferruginea* and metformin have no significant effect on basal blood glucose level (Figure 1) during the 3 hours compared to rats that received distilled water. Glibenclamide significantly (P <0.001) reduced basal blood glucose level, on t_0 we have 70. 3 ± 3.4 mg/dL and 59 ± 2.1 mg/dL, 45 ± 3.8 mg/dL, 31 ± 0.9 mg/dL respectively on t_{60}, t_{120}, t_{180} after substances administration. On the third time we have 47% reduction of basal blood glucose level (Fig 1). A single dose administration of hydro alcoholic extract of *B. ferruginea* and metformin have no significant effect on basal insulin level during the 3 hours compared to that of rats received distilled water. Glibenclamide significantly (P < 0.001) increased basal insulin level, on t_0 we have 0. 09 ± 0.02 ng/mL and 0.12 ± 0.02 ng/mL, 0.163 ± 0.03 ng/mL, 0.166 ± 0.24 ng/mL respectively on t_{60}, t_{120}, t_{180} after substances administration (Fig 2).

Animals were randomly assigned into four groups of 6 each. Group I: The rats received distilled water (10 mL/kg per day) group II: The rats received B. ferruginea hydro alcoholic extract (250 mg/kg), group III metformin 100 mg/kg, Group IV glibenclamide (5 mg/kg). Blood was collected from rats with capillary tube from retino-orbital plexus at 0, 60, 120 and 180 minutes after substances administrations.

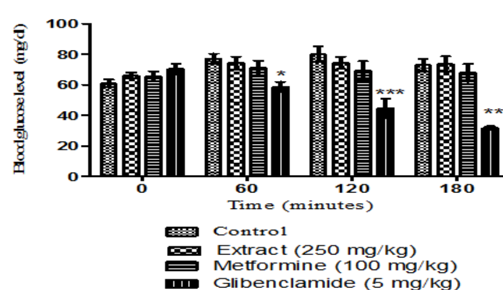

Figure 1: Effect of substances on blood glucose level on healthy rats during 180 min.

The samples were centrifuged at 3000 g for 15 min, the serum obtained was aliquoted and frozen for glucose level assay. The data were expressed as mean ± S.E.M. (*n* = 6) **p< 0.05** P<0.01*** P<0.001 (vs Control)*

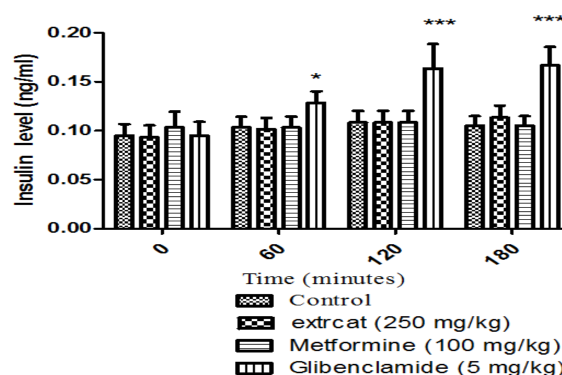

Figure 2: Effect of substances on blood insulin level on healthy rats during 180 min.

Animals were randomly assigned into four groups of 6 each.Group I: The rats received distilled water (10 mL/kg per day) group II: The rats received *B. ferruginea* hydro alcoholic extract (250 mg/kg), group III metformin 100 mg/kg, group IV glibenclamide (5 mg/kg). Blood was collected from rats with capillary tube from retino-orbital plexus at 0, 60, 120 and 180 minutes after substances administrations.

The samples were centrifuged at 3000 g for 15 min, the serum obtained was aliquoted and frozen for insulin level assay. The data were expressed as mean ±S.E.M. (*n* = 6)** p< 0.05** P<0.01*** P<0.001 (vs Control)*

Effect of substances on blood glucose and insulin level on healthy rats during 14 days of administration.

Repeated dose administration of *Bridelia ferruginea* and metformin for 14 days had no effect on basal glucose administration.

We note a significant (P<0.001) decrease of blood glucose levels in animals that received glibenclamide. On D_0 blood glucose level was 72 ± 3.4 mg/dL and decreased to 55.8 ± 2.1 mg/dL on D_7 and 48 ± 1.7 on D_{14} (Fig 3). Repeated dose administration of *B. ferruginea* and metformin for 14 days had no effect on basal insulin level administration.

On the other hand, we note a significant (P<0.001) increase of insulin level in animals that received

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glibenclamide. On D_0 blood insulin level was 0.095 ± 0.015 ng/mL and increased to 0.128 ± 0.041ng/mL on D_7 and 0.135 ± 0.017 ng/mL on D_{14} (Fig 4).

Animals were also randomly assigned into four groups of 6 each, Group I: The rats received distilled water (10 mL/kg per day) group II: The rats received *B. ferruginea* extract (250 mg/kg/day), group III metformin 100 mg/kg/day, group IV glibenclamide (5 mg/kg/day) for 14 day consecutively. Blood was collected from rats with capillary tube from retino-orbital plexus at day 0, 7 and 14 and blood glucose level were determined. The data were expressed as mean ± S.E.M. (n = 6)* *p< 0.05*** *P< 0.01**** *P<0.001 (vs Control) ;*$^{\#}$ *p< 0.05*$^{\#\#}$ *P<0.01 (vs D_0)*

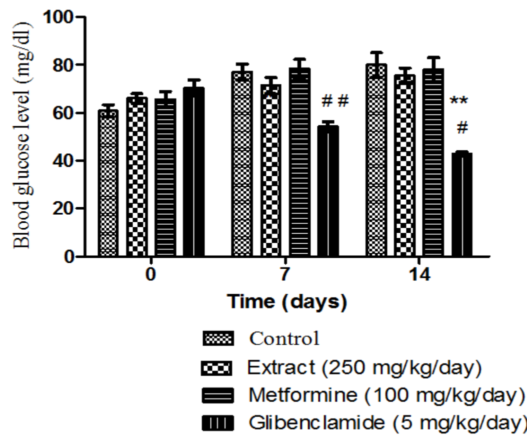

Figure 3: Effect of substances on blood glucose level on healthy rats during 14 days.

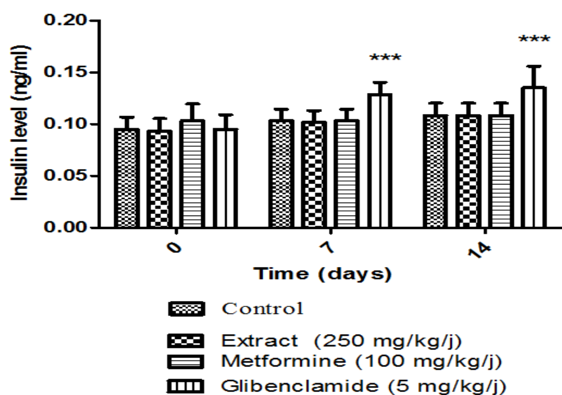

Figure 4: Effect of substances on blood insulin level on healthy rats during 14 days.

Healthy Animals were also randomly assigned into four groups of 6 each, group I: The rats received distilled water (10 mL/kg per day) group II: The rats received *B. ferruginea* ethyl acetate fraction (50 mg/kg/day), group III metformin 100 mg/kg/day, group IV glibenclamide (5 mg/kg/day) for 14 day consecutively. Blood was collected from rats with capillary tube from retino-orbital plexus at day 0, 7 and 14 and blood insulin level was determined. The data were expressed as mean ± S.E.M. (n = 6)**p< 0.05*** *P<0.01**** *P<0.001 (vs Control).*

In vitro toxicological assay

The evaluation of *Bridelia ferruginea* root bark on Vero and N2A cells viability was performed using, neutral red and MTT test assay. *Bridelia ferruginea* showed in N2A cell an IC_{50} of 245 µg/mL for MTT assay and 211 µg/mL for neutral red assay (Fig 5). In Vero cell, we have an IC_{50} of 317 µg/mL for neutral red assay and 286 µg/mL for MTT assay (Fig 6), *Bridelia ferruginea* hydro alcoholic extract was more toxic in N2A cells than Vero cells. Figures show that the extract inhibits cell viability in dose dependent manner.

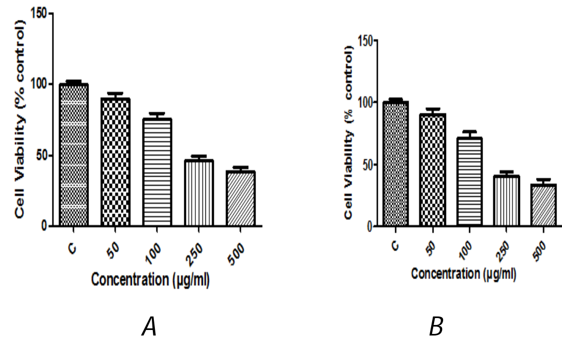

Figure 5: Effect of *Bridelia ferruginea* on the viability of N2A cell lines as measured by: (A) MTT and (B) neutral red uptake assays after 24 h of incubation.

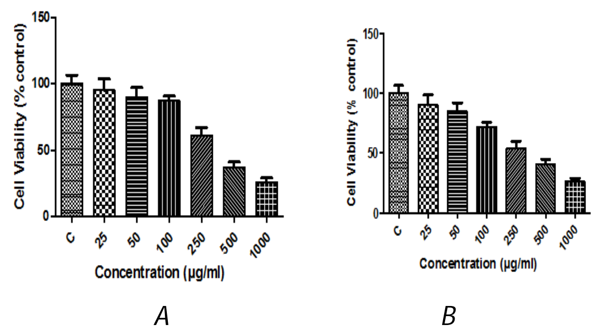

Figure 6: Effect of *Bridelia ferruginea* on the viability of Vero cell lines as measured by: (A) MTT and (B) neutral red uptake assays after 24 h of incubation.

DISCUSSION

The results of this study showed that the administration of *Bridelia ferruginea* hydro alcoholic extract to normal Wistar rats either in single or multiple dose does not lead to a significant reduction in basal blood glucose or insulin secretion in our experimental conditions; during this study, two reference drugs were used. First, metformin which showed no effect on basal blood glucose and basal insulin, is an antihyperglycemic agent. It is well established that metformin has no effect on the basis of blood glucose in normal subjects but reduces the hyperglycemic peak. Its mode of action is multifactorial and includes the increase in glucose utilization by peripheral tissues and inhibition of hepatic and renal gluconeogenesis.[11,12] And secondly, glibenclamide, which induced reduction of basal glucose and increased basal insulin in normal rats; Glibenclamide has an effect on pancreatic level; it stimulates insulin secretion, by binding to a specific receptor (SUR, Sulfonyl Urea Receptor) present on the membrane of the pancreatic beta cells, which inhibits the operation of potassium channels. This

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causes membrane depolarization which produces an influx of calcium into the cytoplasm triggers insulin secretion. Glibenclamide therefore stimulates the release of insulin already synthesized. Cell Sensitivity to glucose is also increased by this molecule.[13,14] This suggests that this extract by reducing hyperglycemia[4] without inducing insulin secretion, has an extra pancreatic effect which would be similar to that of metformin. Then, *Bridelia ferruginea* will be more useful in the treatment of type 2 diabetes in traditional medicine.

The MTT and neutral red assays are two assays commonly used to determine cell viability.[15] MTT is a pale yellow water soluble salt which is converted to a water insoluble highly coloured purple formazan in the mitochondria of living cells. The formazan crystals cannot permeate the cell membrane and accumulate inside healthy cells.[16] The accumulation of formazan is directly correlated to the activity of mitochondria, giving a linear response between cell numbers or activity to the colour intensity. Neutral red, a supravital dye relies on the principle of dye accumulation in the lysosomes and Golgi apparatus of viable, uninjured cells. It does not rely on a reduction reaction to determine viability, thereby reducing the possibility of making type 1 errors (false positives) in the presence of antioxidants or other reductive agents.[8] Therefore both assays were included for the determination of cell viability in the present study. Results indicate that hydro alcoholic extract of *B. ferruginea* tested were not cytotoxic to Vero and N2A cell viability proliferated; the IC_{50} was greater than 100 µg/mL. Any extract whose IC_{50} is > 100 µg/mL was considered not to be cytotoxic.[17] This result confirms the safety of the hydro alcoholic extract of *B. ferruginea* observed in previous toxicological evaluation *in vivo*.[5]

CONCLUSION

In this study, a preliminary demonstration of potential and safety of *B. ferruginea* a medicinal plant used traditionally for treatment of diabetes was established.

Although the antidiabetic activity of *B. ferruginea* root Bark extract and its safety have previously been demonstrated *in vivo*, this work now shows for the first time that extract of this plant act as metformin and confirm its safety *in vitro*. It is envisaged further works on this plant to develop a new affordable and effective phytomedicines for type 2 diabetes.

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